Molecular and Functional Characterisation of the Swiss Drug Clone, a Methicillin-Resistant *Staphylococcus aureus*

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Dedicated to my mom and dad

Zusammenfassung

Ein Methicillin resistenter *Staphylococcus aureus* (MRSA), hat sich in den letzten Jahren unter den Drogenabhängigen der Region Zürich ausgebreitet, der nur eine sehr geringe Methicillin Resistenz mit einem heterogenen Resistenzprofil exprimiert. Im Jahre 2003 konnten 25 % aller identifizierten MRSA Isolate am Institut für Medizinische Mikrobiologie der Universität Zürich diesem endemischen Klon zugeordnet werden. Die Methicillin Resistenz wird in diesem sich verbreitenden Klon durch das *mecA* Gen, welches für PBP2a kodiert, und das auf einem neuartigen staphylococcal cassette chromosome *mec* (SCC*mec*) Element liegt, verursacht. Dieser Resistenzphänotyp führte ursprünglich zu Fehlidentifikation und des weiteren zu Behandlungsversagen. Um seine diagnostische Identifikation zu vereinfachen wurde das SCC*mec* eines repräsentativen „Drogen-Klons" kartiert und teilweise sequenziert.

Viele Faktoren die das Methicillin Resistenzniveau und die heterogene/homogene Expression in klinischen MRSA Isolaten bestimmen, sind noch immer unbekannt. Bei der Suche nach der Ursache der niedrigen Methicillin Resistenz des „Drogen-Klons", wurde eine Punktmutation in der *mecA* Promoter Region gefunden, welche die *mecA* Transkription vermindert. Die Untersuchung der *mecA* Promoter Region, *mecA*-Transkription und PBP2a Menge ergab jedoch, dass das Resistenzniveau in diesem MRSA hauptsächlich durch den chromosomalen Hintergrund bestimmt wird und nur zu einem geringen Mass durch die *mecA* Promoter Mutation, welche die *mecA* Transkription beeinflusst.

Die *mecA*-Induktion wird durch den Repressor und Sensor MecI/MecR1 reguliert, jedoch wird angenommen, dass zusätzliche Faktoren darin involviert sind. Durch ein Screening von Proteinen die an den *mecA* Promoter binden, wurde ein bis anhin uncharakterisiertes DNA-bindendes Protein, mit einem typischen helix-turn-helix Motiv herausgefiltert, das am 5' Ende der *mecA* kodierenden Sequenz bindet. In verschiedenen MRSA Stämmen mit heterogener Methicillin Resistenz resultierte die Deletion dieses DNA-bindenden Proteins interessanterweise in erhöhter Methicillin Resistenz. Da weder *mecA*-Induktion, -Transkription noch PBP2a Menge in den Deletionsmutanten verändert war, muss die modifizierte Resistenzhöhe auf die Regulation von unbekannten chromosomalen Faktoren zurückgeführt werden.

Um chromosomale Faktoren, die für die Methicillin Resistenzhöhe verantwortlich sind zu identifizieren, wurde von diesem „Drogen-Klon" mittels zufälliger Integration eines Transposons eine Bibliothek erstellt und nach Mutanten mit veränderter Methicillin Resistenzhöhe gesucht. Aus 8064 Mutanten wurden 17 Gene identifiziert, die 8 verschiedenen Funktionsklassen zugeteilt werden konnten, und zu einer mehrheitlich reduzierten Methicillin Resistenz führten. Um ihre Funktion in Bezug auf Methicillin Resistenz zu verstehen, sind weitere Untersuchungen nötig.

Summary

Increasing numbers of a methicillin resistant *Staphylococcus aureus* (MRSA) clone were detected in the intravenous drug user community in the area of Zurich. In 2003 25% of all MRSA isolates identified at the Institute of Medical Microbiology, University of Zurich, belonged to this endemic clone. Although this successful clone carries *mecA* encoding PBP2a, which confers methicillin resistance, it expresses very low heterogeneous methicillin resistance causing misidentification and treatment failure. The novel staphylococcal cassette chromosome *mec* (SCC*mec*) of this strain has been mapped and partially sequenced to simplify its diagnostic detection.

Many factors influencing methicillin resistance levels and its heterogeneous/homogeneous expression in clinical MRSA isolates are still unknown. A search for the reason for the extremely low level methicillin resistance of this particular clone, revealed a point mutation in the *mecA* promoter region reducing *mecA* transcription. However, characterisation of the *mecA* promoter region, *mecA* transcription and PBP2a amounts, showed that resistance levels of MRSA were mainly determined by the chromosomal background and only to a small extent by *mecA* promoter mutations affecting levels of *mecA* transcription.

Since factors involved in the pathway of *mecA* induction have not yet been completely identified, and it has been postulated that another factor, in addition to MecI/MecR1 is also involved, a screen for proteins binding to the *mecA* promoter region was performed. This allowed the identification of a previously uncharacterised DNA-binding protein, with a characteristic helix-turn-helix motif, binding to the 5' end of the *mecA* coding sequence. Deletion mutants in different genomic backgrounds of heterogeneously resistant MRSA, exhibited increased methicillin resistance levels. As no interference with *mecA* induction, transcription or translation was detected, the modified resistance levels have to be ascribed to unknown chromosomal factors controlled by this DNA-binding protein.

Construction of a random transposon mutant library, using this low level methicillin resistant clone, allowed identification of a set of new genes affecting methicillin resistance levels. Seventeen new orfs belonging to eight different functional classes were confirmed to alter methicillin resistance levels upon their inactivation. Their relative functions in respect to methicillin resistance still need to be characterised.

1. Introduction

1.1 Staphylococcus aureus

Staphylococcus aureus is a member of the family micrococcaceae, it is a Gram-positive, non-motile, low G-C, facultatively anaerobic coccus, that can behave as both a commensal and a harmful human pathogen. Twenty five to 30 % of healthy people are colonised by opportunistic *S. aureus* in their nasal passages, and on skin and mucous membranes (4, 42). Typical distinguishing features of *S. aureus* include the production of coagulase, clumping factor, catalase and β-hemolysis. In general, the most common diseases caused by *S. aureus* are skin- and soft-tissue infections, impetigo, abscesses, pneumonia, sepsis and foreign body associated infections (70). Two main factors are responsible for the widespread pathogenic success of *S. aureus*, the first is its large range of virulence factors causing a wide range of serious diseases and the second is its strong persistence in hospital settings due to the acquisition of antibiotic resistance determinants especially methicillin resistance, which confers resistance to virtually all β-lactams and their derivatives (4).

1.2 Antibiotics

Antibiotics are substances produced by bacteria or fungi, or synthetic compounds, which at low concentrations have the ability to kill or inhibit the growth of other bacteria/fungi. Antibiotics generally interfere with a much higher affinity to pathways or structures of the bacteria than to target homologues found in the eukaryotic host; thus killing the bacteria while causing no, or little harm to the eukaryotic host. Modification of existing antibiotics can improve their host range, efficacy, and pharmacokinetics. A broad spectrum of antibiotics is available for treatment of all kinds of infections caused by diverse bacteria (121).

Antibiotics can be classified into two categories, according to their bacteriostatic or bactericidal activity in vitro. This classification strongly depends on conditions, such as the bacterial growth rate, bacterial killing kinetics, duration of exposure to the antibiotic, bacterial density, and the organism targeted. However, as the bacteriostatic/bactericidal activity is determined in vitro, the actual efficacy can differ in vivo depending on the growth stage (86). Antimicrobial potency also depends on the bacterial growth conditions. Free growing, planctonic bacteria are affected more easily by bactericidal antibiotics than sessile bacteria that persist within a biofilm. Increased tolerance to antibiotics in sessile bacteria might be partially caused by restricted access of the antibiotic to the cells, as they are enclosed within an extracellular matrix, but is mostly attributed to their slower growth rate, reduced antibiotic up take due to decreased metabolic activity and their altered gene expression. Other factors

suggested to influence resistance in biofilms are oxygen-/nutrient-limitation, pH, and the accumulation of toxic metabolites (17, 26, 94, 103).

Main targets of antibiotics include bacterial peptidoglycan synthesis, DNA replication, transcription, protein synthesis, and folate coenzyme biosynthesis (Figure 1) (122).

Bacteria have, however, struck back by evolving several resistance strategies. Their high mutation rate of $1:10^7$ and their ability to easily take up new resistance determinants, combined with antibiotic selection pressure, drive the emergence of resistant clones (25, 121). The speed and frequency with which resistance arises and spreads varies between different bacterial species and different antibiotics (14). Three main resistance strategies have been identified:

I) Antibiotic exclusion, by active efflux or decreased permeability.

II) Antibiotic inactivation, by enzymatic degradation or modification through phosphorylation, thiol transfer, acylation, glycosylation, nucleotidylation, or ribosylation.

III) Target alteration, by modification of the target site via mutation, or amplification of the target, or acquisition of new resistant targets (108, 121, 128).

Pathogens, using several of these strategies, are accumulating more and more resistances and their multi-resistance is causing treatment failures. To delay resistance emergence four key strategies have been proposed. First, to determine the optimal use of antibiotics and prevent treatment with subinhbitory concentrations; second, to limit and control the use of antimicrobials; third the rotational use of antibiotics; and finally, application of combination therapy to reduce mutation frequencies (80, 108). But there is still a strong need for the development of new antimicrobial substances to ensure continued treatment options for the growing number of multiresistant microorganisms.

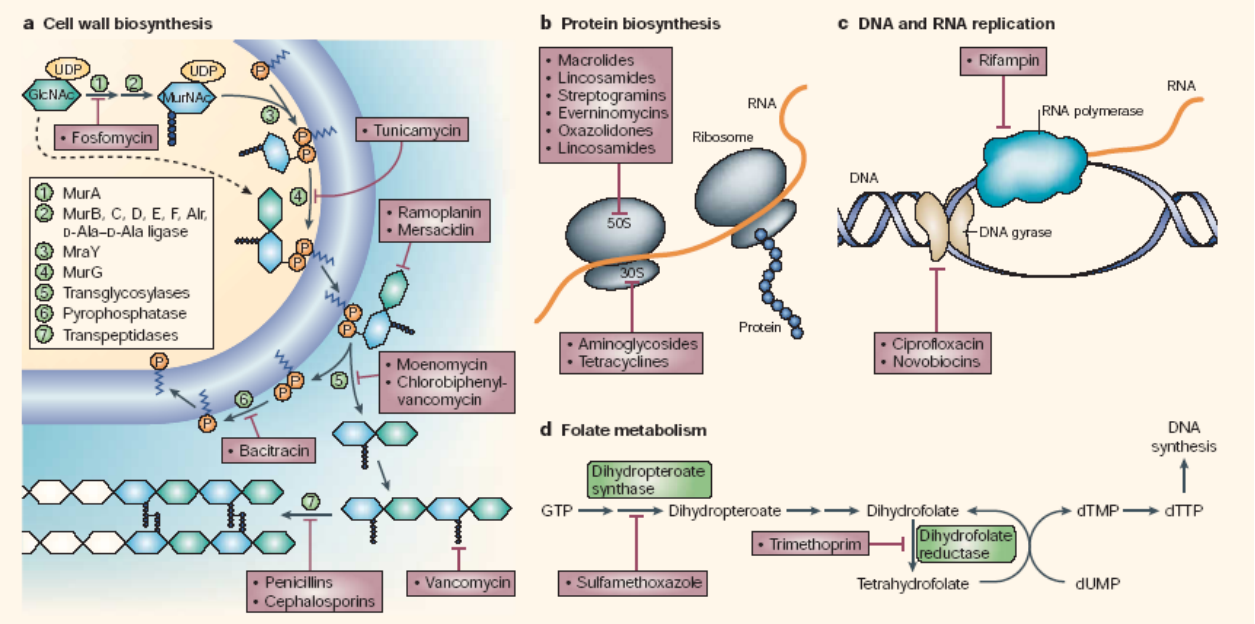

Figure 1.

Schematic overview of antibacterial targets. a, Peptidoglycan biosynthesis. b, Protein biosynthesis. c, DNA and RNA replication. d, Folate metabolism, synthesis of the essential DNA component thymine. Targets of antibiotics are indicated by red lines. Taken from (122).

1.2.1 Cell wall

Cell wall synthesis is a major antibacterial drug target. The cell wall of *S. aureus* consists of 20-50 networked peptidoglycan layers, is ~ 40 nm thick (75) and determines the cell shape and provides the high stability necessary to overcome environmental pressures while maintaining a high flexibility.

The major subcomponent of the staphylococcal peptidoglycan consists of alternating N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) forming a β-1,4-linked disaccharide, with a pentapeptide L-Ala-D-iGln-L-Lys-D-Ala-D-Ala (stem peptide) attached to the MurNAc. The majority of glycan chains have a length of 3 to 10 disaccharide units whereas a small minority of 10-15 % can reach a length of more than 26 disaccharides (12).

In the cytoplasmic steps of peptidoglycan synthesis, fructose-6-phosphate is converted by GlmS, GlmM and GlmU to glucosamine-6-phosphate, glucosamine-1-phosphate and UDP-GlcNAc, respectively. This is followed by the synthesis of UDP-MurNAc from UDP-GlcNAc in two steps by MurA and MurB (Figure 2). The amino acids of the stem peptide are sequentially added to the carboxyl group of UDP-MurNAc by MurC (L-Ala), MurD (D-Glu), MurE (L-Lys), and MurF (D-Ala_2). The α-carboxyl group of the D-Glu is amidated in an ATP- and ammonia-dependent reaction resulting in D-iGln (112). Proteins thought to be involved in the amidation step are GlnA, glutamine synthetase, and its regulator GlnR, as interruption of *glnR* resulted in reduced amidation (43). In the next step MraY transfers the UDP-MurNAc-pentapeptide to the membrane acceptor (undecaprenylphosphate) generating the lipid I precursor. GlcNAc, added by MurG, completes the peptidoglycan monomer subunit called lipid II. Five glycines are then sequentially added at the ε-amino group of lysine by FmhB, FemA and FemB using glycyl-tRNA as donor (56). FmhB is essential and responsible for the incorporation of the first glycine (98), FemA attaches the 2^{nd} and the 3^{rd} glycines and FemB the 4^{th} and the 5^{th} (113). Translocation of the lipid II precursor with its accessory pentaglycine chain to the outside of the membrane is catalysed by a translocase or flippase that has not yet been identified (104).

The disaccharides on the outside of the cell membrane are polymerised by transglycosylation, and the stempeptides are crosslinked by pentaglycine chains connecting L-Lys of the first stem peptide with D-Ala at position 4 of a neighbouring peptidogylcan strand, by transpeptidation (39, 64, 132). It is the cleavage of the terminal D-Ala during transpeptidation that provides the energy necessary to crosslink the stem peptides (64, 104). The pentaglycine is able to crosslink stem peptides of different peptidoglycan strands resulting in a three-dimensional peptidoglycan network with an extremely high crosslinkage degree of 80 - 90% (32, 130, 131).

Transpeptidation and transglycosylation are catalyzed by penicillin binding proteins (PBPs); D,D-acyltransferases all belonging to the serine acetyltransferase group I family. Group II and III members are acetyltransferases with diverse functions, which are either not involved or indirectly involved in peptidoglycan synthesis, or are penicillin-resistant L,D-acetyltransferases, respectively. There are three motifs that are characteristic for the group I family, SxxK, SxN (SxD) and KTG (KSG). *S. aureus* possesses four known group I PBPs; PBP1-3 are classified as high molecular weight (HMW) and PBP4 as a low molecular weight (LMW) PBP. HMW PBPs are subdivided into class A and B. PBPs of group A are bifunctional enzymes and have a non-penicillin binding domain (nPBD) with transglycosylase activity, which is linked to a penicillin-binding domain (PBD) with transpeptidase activity. Group B PBPs also have a PBD as well a nPBD, but the function/activity of this nPBD is still unknown (15, 38, 131).

PBP1 is a class B HMW protein, has a size of approximately 87-kDa, is encoded by *pbpA* and is composed of a domain of unknown function and a transpeptidase domain. It is located at the division site and was shown to be essential for growth and cell division but has no major role in peptidoglycan crosslinking (35, 87, 120).

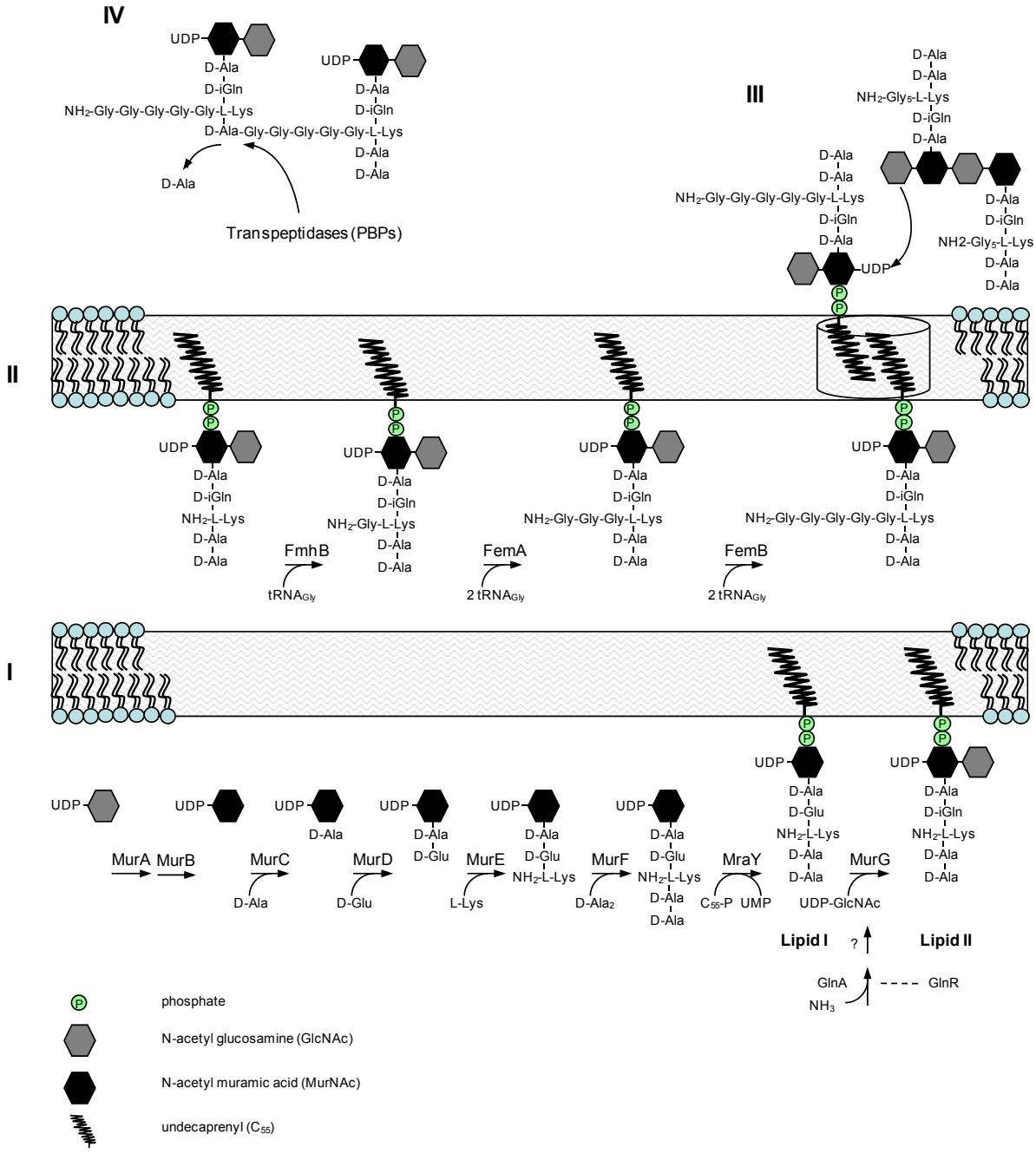

Figure 2.

Peptidoglycan biosynthesis pathway. I, assembly of the lipid II precursor, with MurA as the first catalytic enzyme; II, synthesis of the pentaglycine interpeptide at the lipid II precursor; III, after translocation of the peptidoglycan subunit to the surface, polymerisation of the disaccharide units takes place and IV, crosslinking of the stem peptides via pentaglycines. Adapted from Rohrer and Schneider et al. (97, 105, 112, 124).

PBP2 is a bifunctional 79-kDa HMW, class A PBP encoded by *pbpB*. It is subdivided into a C-terminal transpeptidase domain, and an N-terminal transglycosylase domain which are connected by a small linker. The membrane anchor is attached at the N-terminal transglycosylase domain as shown in Figure 3. These two domains enable polymerisation of the disaccharides and crosslinking of the stem peptides (71, 81, 88). PBP2 has been shown to be localised at the division septum, where cell wall synthesis takes place, and to be essential for cell growth in *S. aureus* (90, 92).

Interruption of *pbpC*, expressing PBP3, a 75-kDa protein, led to a decreased autolytic rate but no other phenotype. Due to its similarity to PBP1, both are class B HMW PBPs with a transpeptidase motif, it was suggested that PBP1 could functionally substitute for PBP3, explaining the lack of a phenotype associated with peptidoglycan composition or crosslinking. Possible functions it could be involved in are septum formation and cell separation (35, 89).

PBP4, encoded by *pbpD* is the only LMW PBP (45-kDa) in *S. aureus*. PBP4 has a C-terminal membrane anchor, and was shown to have D,D-carboxypeptidase, transpeptidase and β-lactamase activity (35, 36, 62). As the transpeptidase activity was found to be nonessential, it is described as a transpeptidase with secondary cross linking activity (131). Peptidoglycan subunits are crosslinked to form up to 17mer multimers; further crosslinking to a higher degree is then executed by PBP4, as interruption of PBP4 results in a reduction in the proportion of highly crosslinked peptidoglycan chains (31, 67, 111). The second activity of a D,D-carboxypeptidase is cleavage of the terminal D-Ala of the stem peptide via hydrolysis, preventing further cross linking of the peptidoglycan chains (132).

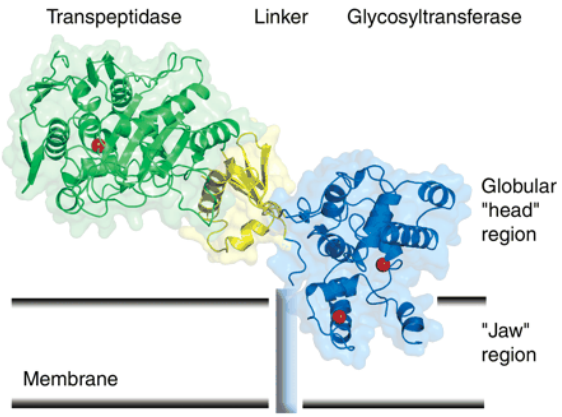

Figure 3.
Structure of the bifunctional PBP2. The transpeptidase domain, the linker and the transglycosylase domain are coloured in green, yellow and in blue, respectively. The putative location of the membrane anchor is also indicated in blue. Active sites are indicated by red spheres. Copied from (71).

1.2.2 β-lactam resistance

1.2.2.1 History

Penicillin, discovered by Sir Alexander Fleming in 1929, was introduced in 1941 to treat life threatening infections caused by *S. aureus*. Only 1 to 2 years later penicillinase resistant *S. aureus* arose in the hospital environment. The prevalence of penicillinase resistant strains increased rapidly within a few years and shortly after penicillin resistant strains also appeared in the community (14, 72, 96). Subsequently, in 1959 the semisynthetic, penicillinase resistant, β-lactam, methicillin was developed. However, the first methicillin resistant *S. aureus* (MRSA) was already detected in 1961 (55) and this new resistance mechanism conferred cross resistance to all β-lactam antibiotics and their derivatives.

1.2.2.2 β-lactam mode of action

β-lactams, including penicillins, cephalosporins, monobactams, and carbapenems, disrupt the balance between peptidoglycan synthesis and degradation thus causing cell lysis (64, 114). β-lactams all have the β-lactam ring structure in common, which is composed of D-valine, L-cysteine and a carbonic acid. Recognition of β-lactams by PBPs is due to the structural analogy between β-lactams and D-Ala-D-Ala of the stem peptide.

The transpeptidase function of native PBPs is blocked by β-lactams which bind to the active site serine. PBPs and β-lactams initially form a reversible noncovalent Michaelis complex, which can either dissociate or undergo acylation to form a stable, covalent acyl-PBP intermediate whose breakdown/deacylation is generally very slow and therefore prevents peptidoglycan crosslinking. In contrast the D-Ala-D-Ala of the stem peptide, the real substrate of the PBPs, is rapidly acylated and deacylated, making the PBPs available for further cross linking reactions (15).

PBP2 is localized at the division septum in growing cells but is dispersed in the presence of β-lactam antibiotics. Acylation of the transpeptidase domain of PBP2 prevents substrate recognition which results in its dissociation from the septum. Localisation seems to be substrate dependent, as blocking or eliminating the substrate also causes dislocation of PBP2 from the septum (90, 91, 104).

β-lactams can have diverse, concentration-dependent effects on bacterial cells. Subinhibitory concentrations of penicillin have been shown to block cell growth, lethal doses to induce bacteriolysis in a cell cycle dependent manner and very high concentrations to result in nonlytic death (37, 74). Recently, Kohanski et. al. (60) provided evidence that lethal concentrations of all bacteriocidal antibiotics, including β-lactams, initiate a common mechanism of cell death. In both Gram-positive and Gram-negative bacteria, inhibitory concentrations of several different classes of antibiotics all disrupted metabolism, leading to

hydroxyl radical formation, which damaged proteins and DNA, resulting in cell death. Sublethal antibiotic concentrations and bacteriostatic antibiotics, however, did not induce this deadly pathway.

Exposure to β-lactams also triggers global changes in transcription, most notably the induction of the cell wall stress stimulon (119). The cell wall stress stimulon is a set of genes upregulated in response to cell wall damage and disruption of stimulon induction abolishes β-lactam resistance (63). Induction of the cell wall stress stimulon requires inhibitory concentrations of β-lactams (76), and is consequently strain-variable as the amount of β-lactam required for induction depends on the strains MIC.

1.2.2.3 Methicillin resistance mechanisms

Different mechanisms have been found to confer methicillin resistance. In borderline resistant *S. aureus* (BORSA) and modified *S. aureus* (MODSA) low level methicillin resistance is expressed by hyperproduction and/or modification of PBPs, respectively (64). The third and by far most common and efficient method is the acquisition of the *mecA* gene encoding PBP2a, a PBP with lower affinity to all β-lactam antibiotics than the cells own PBPs. PBP2a, a class B, HMW, 78-kDa PBP with a C-terminal transpeptidase domain, a N-terminal non-penicillin binding domain and a transmembrane anchor can confer high level methicillin resistance (Figure 4). Strains expressing methicillin resistance due to the acquisition of *mecA*, are referred to as methicillin resistant *S. aureus* (MRSA).

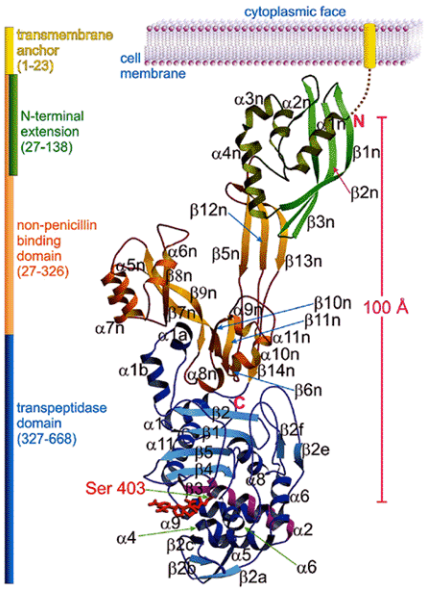

Figure 4.
Structure of the *S. aureus* PBP2a. The different domains are indicated by the colours displayed on the sample board on the left. Yellow: transmembrane anchor; green, N-terminal extension; orange, bilobed N-terminal nPBD domain; blue, C-terminal transpeptidase domain. The active site Ser403 is highlighted in red. Taken from Lim et al. (68).

The low affinity of PBP2a for β-lactams is caused by the inefficient acylation of the active site Ser403, which is distorted and would require conformational changes to be acylated, which is an energetically unfavourable reaction (15, 47, 68).

PBP2a can continue cell wall cross linking in the presence of β-lactams, in cooperation with the uninhibited, essential transglycosylase of PBP2 (Figure 5) (88). Transpeptidation activity of PBP2a is very poor and only muropeptide dimers and some trimers are formed. Nevertheless, this is enough to maintain cell integrity (22). PBP2, which dislocates, from the division septum in methicillin susceptible *S. aureus* (MSSA) in the presence of β-lactams, is maintained in place in MRSA by a functional PBP2a via a proposed protein-protein interaction, as the transglycosylase function of PBP2 is needed for peptidoglycan synthesis (90, 91, 104).

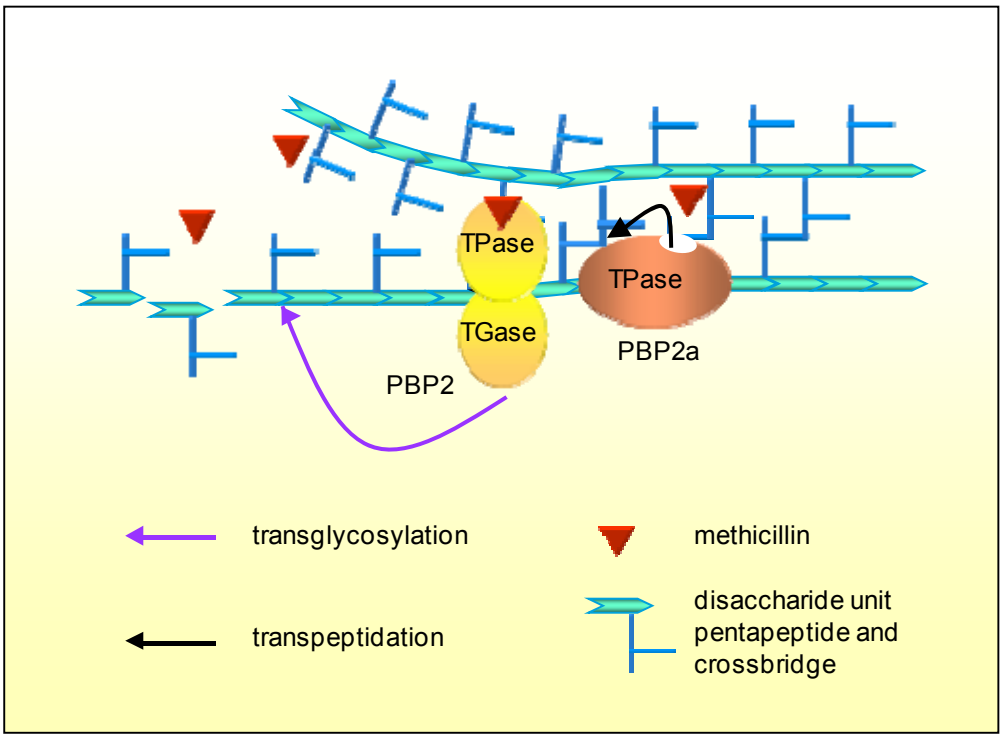

Figure 5.

Scheme showing the cooperation of PBP2a with PBP2 in cell wall crosslinking. In the presence of β-lactam antibiotics the TPase domain of PBP2 is acylated and thereby inactivated. This prevents substrate recognition and results in delocalization of PBP2 from the septal plane. In MRSA strains in the presence of β-lactams the transpeptidase of PBP2a remains active and keeps PBP2 in place. The TPase crosslinking the peptidoglycan stem peptides through the pentaglycine chain and the TGase of PBP2, linking the disaccharide subunits, maintain cell wall synthesis. Adapted from Pinho et al. (88).

1.2.2.4 SCC*mec*

mecA is located on the staphylococcal cassette chromosome *mec* (SCC*mec*) a genomically-integrated resistance island with a proclivity for easily taking up and accumulating resistance determinants (1, 25, 50). The SCC*mec* integrates site- and orientation-specifically into a unique site at 3' end of *orfX*, an open reading frame of unknown function near the origin of replication, called $attB_{SCC}$. After its integration, via cleavage of the $attB_{SCC}$ site, an incomplete direct repeat consisting of two 15-bp DNA fragments is formed (Figure 6) (52). These 15-bp DNA fragments, termed ISS (integration site sequence) appear to be the site recognised by the SCC*mec*'s own recombinases *ccrAB/C* (cassette chromosome recombinase), which are responsible for insertion and excision of the element (52, 57). Composite SCC*mec*s might have evolved by sequential integration of different SCC*mec* elements followed by deletion or recombination events (45, 48, 52, 79). Multiple SCC*mec* excision variants in strains with composite SCC*mec*s have previously been described (27, 54). Depending on the number of ISS, with a consensus of 5'-GANGCNTATCANAANTNN-3', the number of possible excision variants and the corresponding number of elements integrated in tandem into $attB_{SCC}$ can be predicted since each inserted element will be flanked by an ISS (Figure 7). These composite elements can either excise partially or as a whole. In the case of a non recombinase carrying element the *ccrAB/C* of an adjacent element can facilitate its excision. Excision and reintegration of different genomic elements into $attB_{SCC}$ is likely to result in the generation of new SCC*mec* subtypes, increasing the high variability within these elements (54). Spontaneous excision occurs at a very low frequency in the presence of a functional recombinase, therefore down regulation of *ccr* loci might stabilize the SCC*mec* within the chromosome (57). The conditions under which *ccrAB/C* are active have yet to be discovered.
SCC*mec* elements are roughly divided into six main classes according to the combination of ccr and mec complex that they carry. They also differ in size from 21 to 67 kb and in their repertoire of resistance determinants (Table 2, Figure 8) (52, 53, 57, 61, 73, 84). Type I to III SCC*mec* are predominantly found in so called hospital acquired MRSA whereas strains carrying types IV to VI are generally community acquired MRSA (see section 1.3.1) (16, 25, 29, 61).
Five ccr allotypes have been described, four of which contain the two recombinases *ccrA* and *ccrB* and the fifth contains the single gene *ccrC*. The mec complex consists of the *mecA* gene and its divergently transcribed regulatory genes *mecI/mecR1*, which are in some cases deleted/truncated. mec complex A contains an intact regulatory locus (*mecI-mecR1-mecA*) adjacent to *mecA*; class B mec complex (IS*1272-ΔmecR1-mecA*) and C2 (IS*431-ΔmecR1-mec*A), also found in *S. aureus*, have no *mecI* and a *mecR1* truncated either by IS*1272* or IS*431*, respectively. In addition to the mec/ccr complexes and some resistance elements, SCC*mec* DNA is divided into 3 regions, J1 to J3, termed "junkyard" regions as they mainly

contain truncated and nonessential genes of unknown function. Several SCC*mec* variants have been differentiated, based on differences in their junkyard regions (69, 109).

The origin of SCC*mec* is unknown but it is thought to have been horizontally acquired from another species. The closest structural homologue of *mecA* was found in *S. sciuri*, recovered from rodents and primitive mammals, and shared an 88% amino acid sequence identity with *mecA* of *S. aureus* (21, 59, 129). Selection on increasing β-lactam concentrations was used to generate a resistant strain from the susceptible *S. sciuri* carrying the *mecA* homologue. A single mutation in the operator region of the *mecA* homologue was found to be responsible for its increased transcription and resistance levels in *S. sciuri* and allowed this *mecA* homologue to confer methicillin resistance in *S. aureus*. Because this gene was able to confer β-lactam resistance in *S. aureus*, it was suggested to be the evolutionary precursor of *mecA* (129).

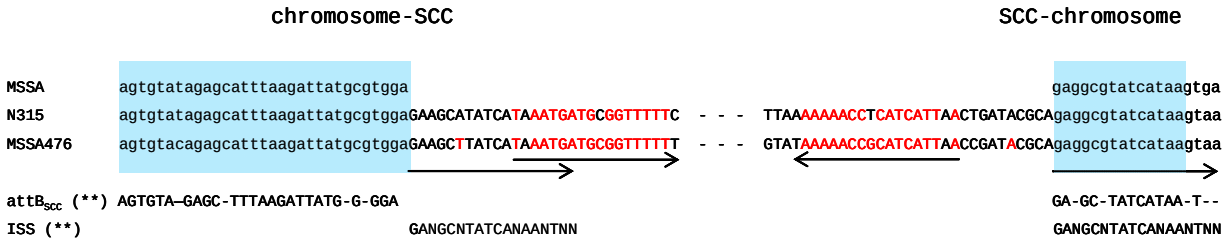

Figure 6.

Chromosome-SCC*mec* junction sequence. The sequences of a MSSA, N315 (MRSA, SCC*mec* type II) and MSSA476 (SCC_{476}) were aligned. *orfX*, a gene of unknown function, indicated by blue shading, contains the attB site where the SCC*mec* element integrates. In MSSA476 a SCC element without *mecA*, was found to have integrated. Direct and indirect repeats are represented by arrows. Consensus sequences of $attB_{SCC}$ and ISS (integration site sequences) were taken from Ito et al. (53).

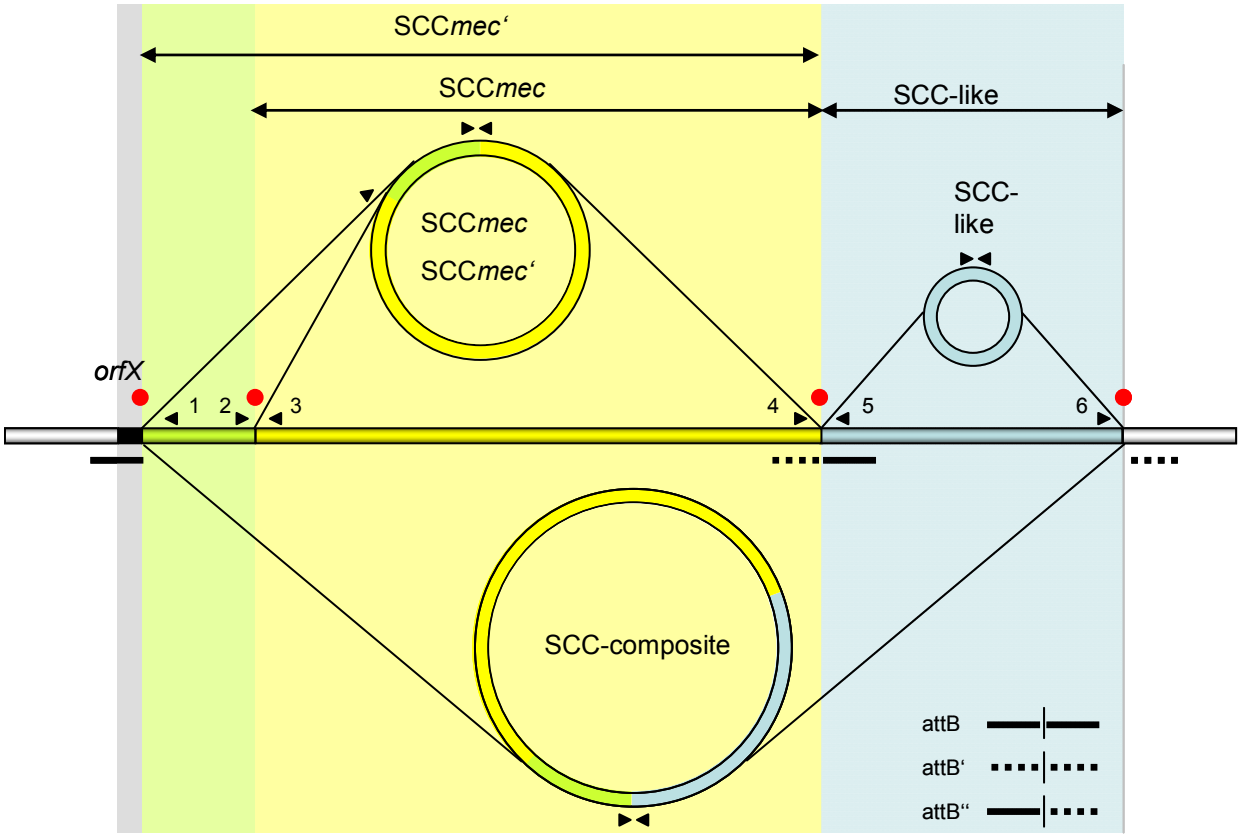

Figure 7.

Excision variants of SCC*mec* described by Jansen et al. (54). Schematic overview of a composite SCC*mec* and its excision/circularization variants in the presence of an active recombinase. Composite SCC*mec* elements arise upon sequential integration of SCC*mec* and SCC-like elements. All of these elements are framed by ISSs, indicated by a red dot, which are recognized by recombinases, enabling excision and circularization of the elements. Primers used to detect circularization products are shown as arrows. The following excision variants were possible: 3-4, 5-6, 1-4, and 1-6; 1-2 and 3-6 were also possible but were not detected. Thick and dotted lines indicate the chromosomal attachment sites which are disrupted upon integration and which are reunited after circularization due to excision of these elements. attB is reconstituted after loss of the SCC*mec* element (1-4, yellow-green). attB’ was found after excision of the SCC-like element (blue) and attB’’ upon excision of the complete composite SCC*mec* element (green-yellow-blue), ligating 1 and 6.

Table 2. Major classes of SCC*mec*, divided by their ccr and mec complexes.

SCC*mec* type	**ccr complex**	**mec complex**	**Size**	**Resistances**
I	1	B	34 kb	Oxa
II	2	A	51 kb	Oxa, Ery, Kan, Tob, Ble
III	3	A	67 kb	Oxa, Ery, Tet, Mer, Cad
IV	2	B	21-24 kb	Oxa
V	5 (ccrC)	C2	28 kb	Oxa
VI	4	B	~ 22 kb	Oxa

Abbreviations: Ble, bleomycin; Cad, cadmium; Ery, erythromycin; Kan, kanamycin; Mer, mercury; Oxa, oxacillin; Tob, tobramycin.

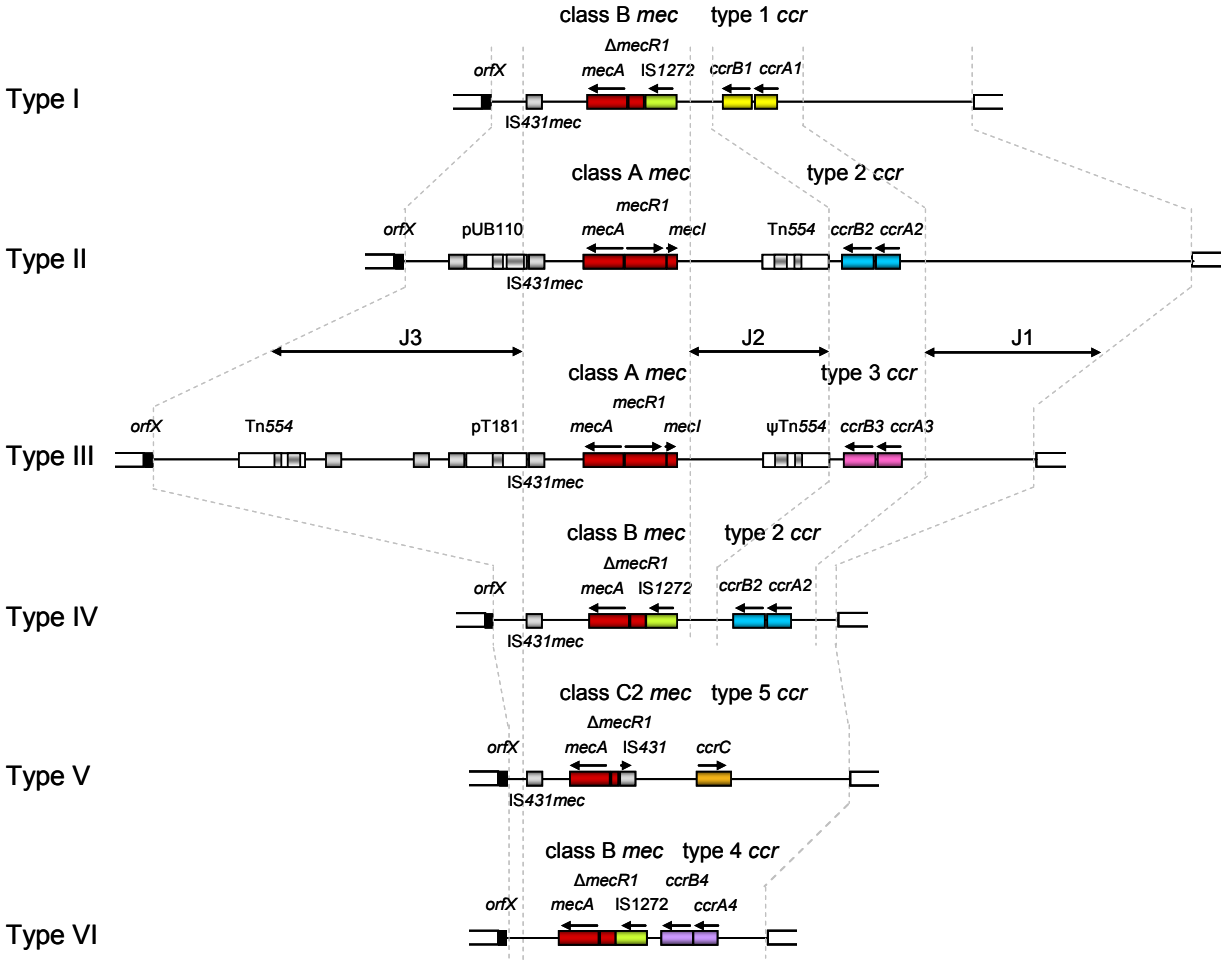

Figure 8.

Basic structure of all major SCC*mec* types. The combination of ccr complex and mec complex is the main factor determining the class of SCC*mec* element. Other attributes include insertion sequences, transposons, integrated plasmids and the size of the elements. Subtypes are typed according to their J-regions (J1, J2, J3). These J-regions are indicated by arrows and dashed lines. The white bars are chromsomal regions outside of the SCC*mec*, and *orfX*, the SCC element integration site, is shown in black. Additional resistance are encoded by Tn554: erythromycin resistance; ψTn554: cadmium resistance; pT181: tetracycline (58); pUB110; kanamycin, tobramycin and bleomycin resistance (77). Adapted from (53, 83, 85).

1.2.2.5 Regulation of *mecA*

mecA, the gene producing PBP2a, is controlled by the divergently transcribed regulatory elements, *mecI*, encoding a repressor protein and *mecR1*, a transmembrane β-lactam sensor transducer (49, 107). A structurally and functionally similar regulatory system controls the penicillinase gene *blaZ*, which is divergently transcribed from its regulators *blaR1* and *blaI*, also encoding a sensor transducer and repressor, respectively. BlaI and MecI bind as homodimers to their corresponding promoter-operator regions, preventing *blaZ/mecA* transcription, as well as their own and *blaR1/mecR1* expression due to the overlapping promoter regions (102). In the presence of β-lactams the sensor domain of BlaR1/MecR1 is activated and transmits a signal from the extracellular domain to the cytosolic domain, leading to autoproteolytic cleavage and activation of the N-terminal BlaR1 domain, which proteolytically cleaves the repressor causing dissociation of BlaI/MecI from the promoter-operator region. Transcription of *blaZ/mecA* and their respective regulatory loci then proceeds. The repressor proteins, BlaI/MecI, have an amino acid sequence identity of 61% (33) and as homologs with structural and functional similarity they can functionally substitute for each other. In strains that constitutively produce *mecA*, due to missing or truncated *mecR1/mecI* regulatory elements the introduction of *blaI/blaR1* results in controlled *mecA* expression. Reciprocally, *blaZ* can also be regulated by *mecI/mecR1*. However, while the repressors can replace each other, the sensor transducers, with an overall amino acid identity of 34% (125), are specific and only able to cleave their own repressors (78).

BlaI/MecI, contain a DNA binding and dimerisation domain and bind specifically as dimers to the promoter-operator region of *blaZ/mecA.* In the *blaZ* promoter the dimerized repressors bind to two 18-bp palindromes (R1 and Z dyad) which are separated by 13 bp, while in the *mecA/mecR1* intergenic region they bind to a 30-bp palindrome with two complementary 15-bp sections (Figure 9). Each half of the palindrome contains an inverted repeat of 2x 4 bp (TACA/TGTA) that is protected by BlaI/MecI binding (102, 107), (18). The distance between the binding sites of the protein dimers determines the binding orientation relative to the DNA. At the single palindrome of the *mecA* promoter the dimers bind on opposite sides to each other due to steric hindrance, whereas at the *blaZ* promoter the dimers can bind next to each other on the identical DNA axis due the 13-bp gap between the two palindromes, each of which carries an inverted repeat (Figure 10) (102).

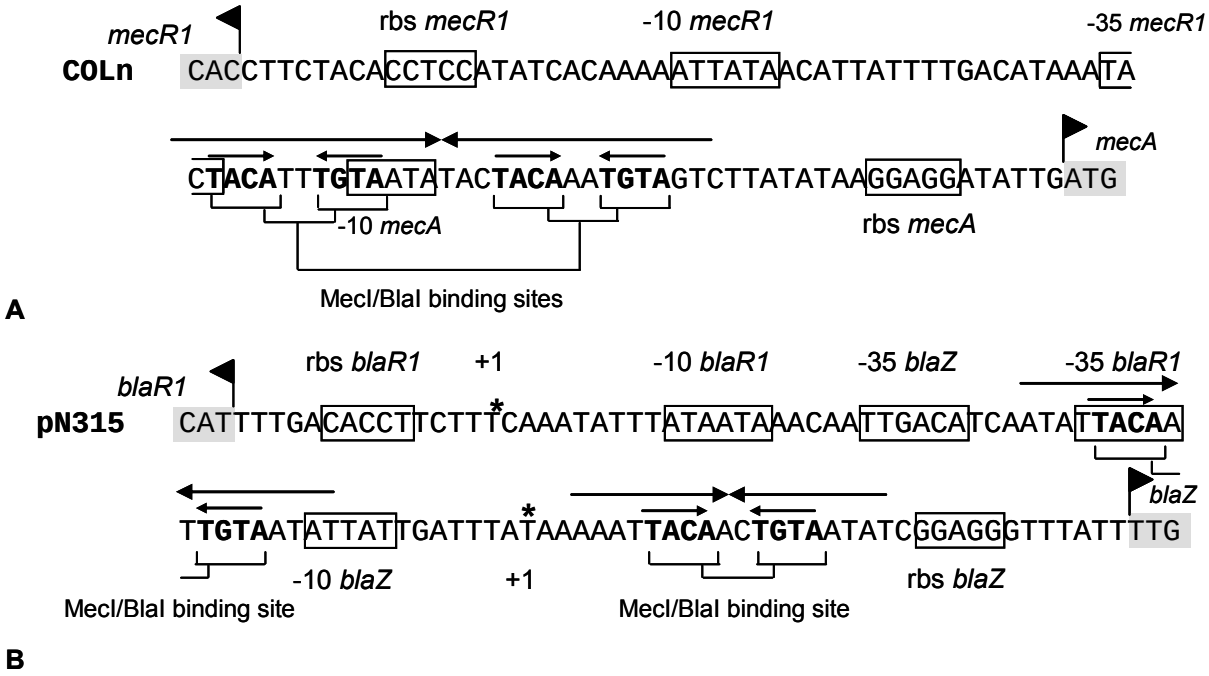

Figure 9.

MecI/BlaI binding sites at the promoter-operator region of A, *mecA* and B, *blaZ*. MecI/BlaI binding sites (TACA/TGTA) are highlighted in bold. Additional features of the promoters indicated are: The ribosomal binding sites (rbs), -10 and -35 boxes which are framed; direct and indirect repeats are shown by arrows; start codons are underlayed with grey and the direction of transcription is shown by flags; transcriptional start sites of *blaZ/blaR1* are indicated by asterisks. Adapted from (18, 41, 102).

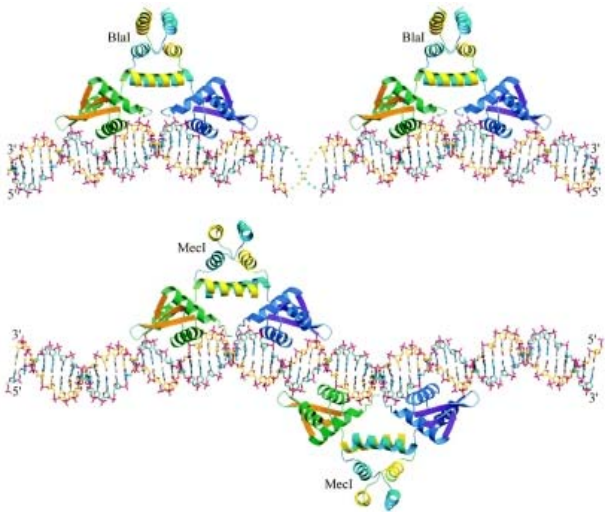

Figure 10.

Scheme of repressor binding to the promoter-operator regions. The protein structure is illustrated as ribbons. The N-terminal helices, the β-strands and the C-terminal helices of one monomer are coloured green, orange and yellow, respectively. In the second monomer the corresponding secondary structures are coloured in blue, magenta and cyan, respectively. Taken from Safo et al. (102).

A, two BlaI homodimers bound to the blaZ and blaR1 dyad of the intergenic region - *blaZ/blaR1*. The spacing between the protein binding sites, allows repressors to bind next to each other.

B, putative MecI-*mecA* complex. Protein homodimers are bound on the opposite sides of the promoter DNA.

1.2.2.6 Signal transduction

The sensor transducer proteins MecR1/BlaR1 sense the presence of β-lactams, which are not able to penetrate through the bacterial membrane, through an extracellular domain which transduces a signal to the cytoplasm to activate the resistance mechanism. BlaR1 consists of a C-terminal penicillin binding domain linked to 4 hydrophobic transmembrane domains, connected via 3 loops (L1-L2-L3) with L2 located extracellularly and L1/L3 in the cytoplasm. L3 contains a metalloprotease domain which induces the defence mechanism (46, 125, 133). When uninduced the C-terminal sensor domain of BlaR1 is non-covalently bound to L2, the loop connecting the transmembrane domains 2 and 3 (Figure 11A) (44). β-lactam then binds to the extracellular sensor domain of BlaR1 forming a preacylation complex which is subsequently acylated by Ser^{389}, the nucleophil which is activated by the carboxylated Lys^{392} known to promote acylation. A significant conformational change, interrupting the non-covalent binding of the sensor domain with loop L2 (40, 44) results in destabilisation of the N-carbamate of Lys^{392} and N-decarboxylation (115). Thus the acylation step as well as conformational changes are irreversible and the sensor is kept in its activated state (8, 40, 115, 125).

The BlaR1 zinc metalloprotease motif ($H^{201}EXXH$), in its cytoplasmic domain, is essential for its own cleavage and for the cleavage of BlaI. Autocatalytic cleavage of BlaR1, induced by binding of β-lactam to its sensor domain, at the $KKSLIKR^{293}RLINIKEA$ of the cytoplasmic domain between R^{293} and R^{294} activates BlaR1 (133), which cleaves BlaI either directly or via an unknown intermediary chromosomal co-factor, such as the postulated BlaR2 (20).

C-terminal cleavage of BlaI, a 14-kDa protein, generates 11-kDa and 3-kDa fragments carrying the DNA-binding and dimerisation domains, respectively (41). The cleavage site is in NH_2-$KSLVLN^{101}F^{102}AKNEELNN$ between N^{101} and F^{102} leaving FAKNEELNN as the amino acid end sequence of the cleaved C-terminal fragment. Mutational substitution of N^{101} or F^{102} prevents proteolytic cleavage of BlaI and therefore prevents derepression of *blaZ* transcription (Figure 11B) (133).

Signal transduction via MecR1 is similar to BlaR1, but one major difference is their differential interaction kinetics with β-lactams (13). Induction of *blaZ* transcription and penicillinase expression requires minutes whereas induction of PBP2a expression over MecR1 takes hours (78, 100). The reversible preacylation complex formed between β-lactams and MecR1 is less efficient and the subsequent acylation reaction is slower than that of BlaR1. The slower sensing of β-lactams due to its poor interaction kinetics might be the reason for the slow derepression of *mecA* by MecR1 (13).

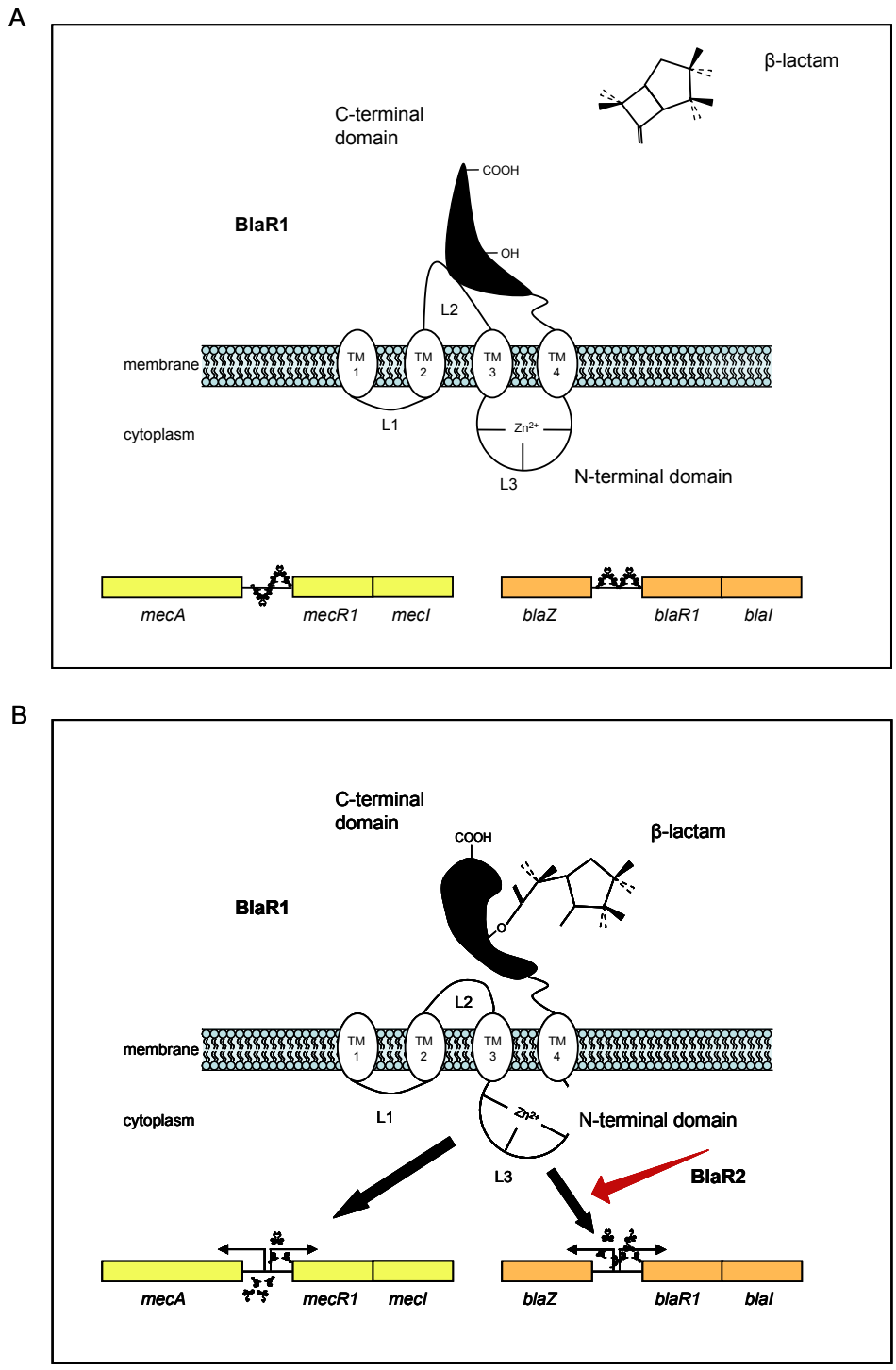

Figure 11.

Schematic regulatory system of BlaR1/BlaI/BlaZ. A, *mecA*, *blaZ* and their divergently transcribed regulatory genes *blaR1/blaI* and *mecR1/mecI* are repressed by BlaI homodimers. B, BlaR1 undergoes acylation in the presence of β-lactam molecules. Acylation causes a conformational change in the C-terminal BlaR1 domain activating a signal transduction pathway, promoting proteolytic cleavage of the N-terminal BlaR1 domain, followed by cleavage of BlaI, either directly by BlaR1 or indirectly by BlaR2, resulting in derepression of *mecA-mecR1/mecI* and *blaZ-blaR1/blaZ*. Structural genes of *mecA/mecR1/mecI* are coloured yellow and *blaZ/blaR1/blaI* orange. Adapted from Thumanu et al. (115).

1.2.2.7 *fem/aux* factors

The majority of MRSA strains express methicillin resistance heterogeneously. In heteroresistant strains most of the cell population is capable of expressing low level resistance and only a small subpopulation is able to grow at high antibiotic concentrations. The segregation of highly resistant subclones occurs at quite a high frequency, above the rate of spontaneous mutations (99, 101). The genetic mechanisms controlling methicillin resistance levels are largely unknown. However, a factor which might contribute to the expression of heterogeneous methicillin resistance was described recently. The deletion of the postulated sodium-dependent symporter homologue SA0501 converted a heterogeneous methicillin resistant strain into a homogeneous resistant strain at elevated temperatures (110).

Chromosomally encoded factors seem to be the main effectors determinating the resistance level of staphylococcal strains carrying *mecA,* as PBP2a expression levels do not directly correlate with methicillin resistance levels (5, 7, 24). Genes outside the SCC*mec* element that influence methicillin resistance levels are referred to as *fem*, factors essential for methicillin resistance or *aux* for auxiliary genes (7, 23, 24).

fem/aux factors include genes involved in cell wall biosynthesis, turnover or regulation (6), protein kinases, ABC transporters, and the catabolite control protein CcpA (24, 106). *mecA* transcription was not reported to be altered by interruption of any of the *fem/aux* factor except for by *murE*. But it has not been shown whether *mecA* transcription is directly or indirectly regulated by *murE*.

1.3 Clinical significance of MRSA

MRSA are not only a major threat because of their β-lactam resistance but also because of their high adaptability under selection pressure. Penicillin and methicillin resistance were acquired within a short time period and other non-β-lactam antibiotic resistance determinants can easily be taken up by the integration of plasmids or transposons into their SCC*mec* element (1, 25). Multi-resistance has severely limited available treatment options for MRSA.

Vancomycin has become one of the antibiotics of last resort for treating multiresistant MRSA. Glycopeptides, such as vancomycin disturb cell wall biosynthesis by irreversible binding to the D-Ala-D-Ala of the peptidoglycan stem peptide preventing peptidoglycan crosslinking. Vancomycin was used for over 30 years before resistance was detected in MRSA, then within the last 10 years both low-level vancomycin-intermediate resistant (VISA) and high level vancomycin resistant (VRSA) *S. aureus* have emerged. In VISA, resistance is associated with a thickened cell wall and reduced cell wall crosslinking which leads to an increased number of free D-Ala-D-Ala in the cell wall, which act as false targets for

vancomycin and prevent it from reaching its lethal target (111, 122). In VRSA, acquisition of the *vanA* operon of *Enterococcus faecalis* results in synthesis of an altered peptidoglycan stem peptide, changing the terminal D-Ala-D-Ala to D-Ala-D-Lac, which has a strongly reduced affinity for vancomycin (3, 72). In three of four described VRSA cases, vancomycin resistant *Enerococcus faecalis* carrying the v*anA* gene could also be isolated, indicating that horizontal transfer of *vanA* has occurred (3).

MRSA strains with heterogeneous β-lactam resistance profiles have proven to be a huge problem for detection in diagnostics. Because their population is composed of a large portion of low level resistant and only a small fraction of high-level resistant subclones, they can appear to be MSSA, according to the CLSI guidelines (19), using traditional susceptibility testing. β-lactams commonly used for infection treatment, will then select for proliferation of highly resistant sublcones, causing treatment failure. In the Netherlands an increasing number of heterogeneously low-level resistant strains were detected. This might also be the case in other countries, and is an alarming situation which needs to be carefully monitored (51, 123, 127).

1.3.1 Community and hospital acquired MRSA

Community acquired MRSA (CA-MRSA) have only emerged as a major problem in the last few years, whereas hospital acquired MRSA (HA-MRSA) have been prevalent since the 1970's, soon after introduction of methicillin, probably due to their continual exposure to antibiotic selection pressure (1, 55). CA-MRSA are described as a class of MRSA causing infections in the "community settings", where patients have not been exposed to classical risk factors such as recent hospitalisation, surgery, residence in a long term care facility, dialysis and indwelling percutaneous medical devices and catheters (82). Comparison of CA-MRSA with HA-MRSA showed a higher prevalence of skin and soft tissue infection due to CA-MRSA than to HA-MRSA, in which infections of the respiratory or urinary tract were more frequent. CA-MRSA were generally found in younger people and had a high carriage of staphylococcal enterotoxins A, C, K, and staphylococcal chromosome *mec* type IV or in some cases type V (34). Panton Valentine leukocidin, previously used as an indicator for CA-MRSA, has been shown to be present in only 20 % of all CA-MRSA analysed and can also be carried by HA-MRSA. HA-MRSA on the other hand were more likely to produce superantigenic toxins (TSST-1, enterotoxins) and typically carried SCC*mec* types I to III, with SCC*mec* type II being the most predominant (30, 82, 118). Another factor discriminating between these two MRSA classes is their growth rate. In general CA-MRSA are much faster growing (83).

1.3.2 Clonal diversity

According to multi locus sequence typing (MLST), most successful MRSA clones belong to one of five main clonal complexes (CC) CC5, CC8, CC22, CC30, CC45, indicating a low frequency of SCC*mec* acquisition by only a few MSSA strain lineages (95, 118). SCC*mec* type IV has been found in twice as many diverse clonal lineages than any other SCC*mec* type. Its small size, which might elevate the transfer efficiency or reduce fitness costs upon its acquisition compared to other SCC*mec* elements, might favour type IV SCC*mec* acquisition (83, 95). Representative, successful epidemic MRSA clones spreading worldwide causing disease are the Iberian, Brazilian, Hungarian, New York/Japan, pediatric and EMRSA-16 clones (1).

1.3.3 MRSA distribution/prevalence

In a European-wide study between 1999-2002, with 27 countries participating, a north-south gradient was detected with low MRSA prevalence in the north and high in the south, ranging from 0.5 to 44 % (Figure 12). During these three years significant increases were found in Belgium, Germany, Netherlands, Ireland, and the UK. In the 2005 annual report from EARSS (European Antimicrobial Surveillance System), MRSA prevalence in Belgium, Germany, Netherlands, Ireland, and the UK was stable or showed only a slight increase between 2002 and 2005. In this time period Finland and Denmark had significant increases in prevalence but still had low overall incidences of 2.91 % and 1.7 %, respectively. In 2002 only four countries had over 40 % MRSA, whereas in 2005 twice as many exceeded 40 %. Interestingly, the MRSA frequency decreased in Slovenia showing that the trend can also be reversible (2, 116).

Figure 12.
Geographical region investigated for MRSA prevalence (1999-2000) taken from (116). Different proportions are indicated by the grey scale.

Switzerland. In a one year epidemiological study (1997), *S. aureus* isolates of five University hospitals and some additional hospitals/rehabilitation institutions were investigated. In Geneva, MRSA were found to be endemic but in general the MRSA prevalence in Switzerland is low with an average value of 2.8 cases/10'000 admissions. Basel, Bern, Geneva, Lausanne and Zurich University hospitals were found to have 4, 3, 23, 3 and 6% MRSA, respectively. These MRSA statistics show that variation in prevalence is not only found between countries but also between different geographical areas within a country. Of four predominant clones identified, three were mainly found in Geneva whereas the 4th clone termed WCH, identical to the Belgium clone 2 and present in Switzerland since 1995, was found in various institutions. Therefore, there is a great diversity found between MRSAs but some clones appear to be able to spread and persist better than others (10, 11).

West-Switzerland. In an 8 year survey from 1997-2004 in west Switzerland, changes in clone frequencies, clone replacements and emergence of new clones was observed. Although there was a rapid change in the clone population, the overall rate did not change over time. Four predominant clones, related to other European clones, the Japan/New York, Southern Germany, Iberian and Berlin clone were present in this area. The most frequent SCC*mec* types found in this study, were type IV and II, representing both CA- and HA-MRSAs (9).

Zurich. The prevalence of the different SCC*mec* types of MRSA in the area of Zurich in 2003 was analysed. Proportions of SCC*mec*s were 3.3 % type I, 10% type II, 12.2 % type III, 45.6 % type IV, 24.4 % SCC*mec*$_{N1}$ ((27), see section 3.1), 1.1 % SCC*mec*$_{ZH47}$ ((48), see section 5.3) and 3.3 % had untypable SCC*mec*s. In this study, strains carrying SCC*mec* type IV and N1 were the most prevalent. By multi locus sequence typing (MLST), four predominant clones were identified: ST217-CC22 similar to EMSA-15; ST225-CC5 related to the Japan/New York clone; ST613-CC8, a new clone; and ST45-CC45 found among the drug user community in Zurich which is the same genetic background as the Berlin clone ((93), see section 5.3). Two clones were found to be predominant in Zurich and in the western part of the country, the Japan/NewYork clone and the Berlin clone.

1.3.4 Emergence of new epidemic MRSA

New epidemic heterogeneously resistant MRSA with similar phenotypes emerged from 1992 onwards in Germany, France, Finland and Australia. These clones were resistant to β-lactam antibiotics but carried no other resistance markers and went on to replace multidrug resistant MRSA in hospital settings (66, 117, 126). In a seven years surveillance study of French hospitals a clear increase in non-multiresistant MRSA, gentamicin susceptible MRSA (GS-MRSA) occurred, while the overall prevalence of MRSA remained relatively stable. Antibiotic resistance profiles showed increased susceptibility to kanamycin, tobramycin, lincomycin, pristinamycin, tetracycline, minocycline, co-trimoxazole, rifampin and fusidic acid but higher frequencies of chloramphenicol resistance (66). Two variants, one with high fitness and short generation time similar to MSSA strains and the second with reduced fitness and longer generation times, like multiresistant MRSA, were found, and the prevalence of the fitter strain was significantly higher. Decreased fitness upon acquisition of resistance and an inverse relationship between resistance levels and fitness, has previously been described (28, 65). Antibiotic resistance determinants, resistance levels and a strains genetic background can all determine a clones fitness and influence its predominance or disappearance (65).

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2. Aim of this study - Project description

The Swiss drug clone, represented by strain CHE482, is a low-level methicillin resistant *Staphylococcus aureus* (MRSA) found spreading in the drug user community of Zurich, Switzerland. Strains belonging to this clone are predestined to be misinterpreted as methicillin susceptible *S. aureus* (MSSA), due to their low and heterogeneous methicillin resistance, leading to treatment failure. Molecular characterisation of this strain would allow fast and accurate identification of this clone preventing misidentification, and should give additional information about *mecA* regulation and factors affecting β-lactam resistance. To do so, four projects were undertaken.

Project I: Variability in SCC*mec*$_{N1}$ spreading among injection drug users in Zurich, Switzerland. (published, Ender et al. BMC Microbiology)
A commonly used multiplex PCR, used to type SCC*mec* elements of MRSA strains, failed in strain CHE482 and isolates of the same clone, indicating the existence of a novel SCC*mec* element. The aim of this project was to identify the recombinase genes, mec complex and additional resistance determinants of this new SCC*mec* and to determine the end sequences and create a map of this element, in order to monitor its spread and development.

Project II: Impact of *mecA* promoter mutations on *mecA* expression and β-lactam resistance levels. (accepted by International Journal of Medical Microbiology)
Genetic factors influencing β-lactam resistance levels, and hence potentially responsible for the low β-lactam resistance in CHE482, are unknown. Sequencing of the *mecA* gene and promoter sequence identified a promoter mutation in the -10 box of the intergenic region of *mecA/mecR1*, creating a perfect palindrome. The aim of this project was to characterize the impact of this one nucleotide substitution on β-lactam resistance levels, since the MecI and/or BlaI repressors controlling *mecA* transcription bind to this palindromic region. Comparison of wildtype and mutant promoters, by means of resistance testing and transcriptional and translational analysis of *mecA*/PBP2a in different genetic backgrounds, showed decreased resistance, lowered *mecA* transcription, and lowered PBP2a production from the mutated promoter compared to wildtype promoter. Nevertheless, expression of the *mecA* preceded by the various *mecA* promoter variants in two *S. aureus* of different genetic background showed that the impact of the promoter mutation on resistance levels was smaller than the contribution of the strains genetic background, suggesting that β-lactam resistance levels are mainly controlled by chromosomal factors.

Project III: A novel DNA-binding protein modulating β-lactam resistance in *Staphylococcus aureus.* (manuscript in preparation)

In addition to the MecI/MecR1 or BlaI/BlaR1 control of *mecA* transcription, other hypothetical factors involved in *mecA* regulation have been postulated. A possible target for regulatory elements is the *mecA/mecR1* intergenic region, where the repressors MecI/BlaI bind. A search for additional proteins binding to the *mecA/mecR1* intergenic region identified a novel protein with DNA-binding capacity. Construction of deletion mutants in heterogeneously methicillin resistant MRSA isolates of different backgrounds resulted in increased resistance, while inactivation in a homogeneously resistant MRSA decreased β-lactam resistance. However, no changes in *mecA* transcription levels and PBP2a amounts were observed. This suggests that this newly identified DNA-binding protein affects methicillin resistance levels indirectly over the control of other genomic factors.

Project IV: Random transposon insertion library of CHE482, a methicillin resistant *Staphylococcus aureus* (MRSA): Identification of novel *fem* factors.

mecA, encoding PBP2a, is essential for expression of β-lactam resistance but the level of resistance is determined in a large part by the strains genomic background, by so called *fem/aux* factors. Several chromosomal factors involved in peptidoglycan synthesis, hydrolysis, global gene regulation, teichoic acid synthesis and genes of unknown functions, have already been identified as *fem/aux* factors. To extend this list, a random transposon library of the low level resistant CHE482 was constructed. The library consisting of 8064 mutants, representing a 2.8 fold coverage of the chromosome, was screened for mutants with decreased and especially increased resistance. Subsequently, 17 new, and 4 already published orfs could be identified and confirmed to influence β-lactam resistance. However, only two insertions resulted in a slightly higher β-lactam resistance.

3 Results

3.1 Project 1

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Variability in SCC*mec*$_{N1}$ spreading among injection drug users in Zurich, Switzerland

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Abstract

Background: An extremely low level methicillin resistant *Staphylococcus aureus* (MRSA) belonging to ST45, circulates among intravenous drug users in the Zurich area. This clone can be misinterpreted as an MSSA by phenotypic oxacillin resistance tests, although it carries a staphylococcal cassette chromosome *mec* (SCC*mec*) element encoding a functional *mecA* gene and it produces PBP2a.

Results: This clone carried a new 45.7-kb element, termed SCC*mec*$_{N1}$, containing a class B *mec* complex (*mecA*-Δ*mecR1*::*IS1272*), a truncated Tn*4003* harbouring the *dfrA* gene, and a *fusB1* gene, conferring methicillin, trimethoprim and low level fusidic acid resistance, respectively. In addition to the two insertion site sequences (ISS) framing the SCC*mec*, a third ISS (ISS*) was identified within the element. SCC*mec*$_{N1}$ also harboured two distinct *ccrAB* complexes belonging to the class 4 subtype, both of which were shown to be active and to be able to excise the SCC*mec*$_{N1}$ or parts thereof. Slight variations in the SmaI-PFGE pattern of the clinical MRSA isolates belonging to this clone were traced back to differences in the sizes of the SCC*mec* J2 regions and/or to a 6.4-kb deletion extending from ISS* to the right end ISS. This latter deletion led to a variant right SCC*mec*-chromosomal junction site. MRSA clones carrying the shorter SCC*mec* with the 6.4-kb deletion were usually ciprofloxacin resistant, while strains with the complete SCC*mec*$_{N1}$ were co-trimoxazole resistant or had no additional resistances. This suggested that the genetic backbone of the host *S. aureus*, although identical by PFGE pattern, had at some stage diverged with one branch acquiring a sulfonomide resistance mutation and the other ciprofloxacin resistance.

Conclusion: This description of the structure and variations of SCC*mec*$_{N1}$ will allow for quicker and easier molecular detection of this clone and monitoring of its spread.

Background

Injection drug user (IDU) populations throughout certain areas of Europe and North America have become major risk groups associated with the epidemic spread of methicillin-resistant *Staphylococcus aureus* (MRSA) [1-5]. The transmission of MRSA clones through both community- and healthcare-associated routes is responsible for the high incidence of soft tissue infections and increases in severe infections such as endocarditis and bacteremia in IDUs [6-8]. Such a clonal dispersal led to MRSA becoming endemic in the Zurich IDU population, where in 2003 24% of all MRSA isolates collected at the University hospital of Zurich belonged to a single, so called "drug clone" [9]. Dissemination of this clone to IDU populations in

BMC Microbiology 2007, **7**:62 http://www.biomedcentral.com/1471-2180/7/62

other, geographically distinct regions of Switzerland has also been recently reported, indicating that it has a capacity for spreading and colonizing new populations [10].

Clinical detection of MRSA can be complicated due to vast strain-to-strain differences in the expression of methicillin resistance. Difficulties arise from strains expressing low-level but heterogenous resistance, that upon exposure to β-lactams segregate highly resistant subpopulations resulting in therapy failure [11]. Misdiagnosis of such strains with very low, phenotypically susceptible, minimum inhibitory concentrations (MICs) is a major problem necessitating the use of molecular detection methods.

Epidemiological classification of MRSA strains is important for monitoring their prevalence and spread, and relies on molecular typing of both their core genomic background and the type of staphylococcal cassette chromosome *mec* (SCC*mec*) they harbour. SCC*mec* is the chromosomally integrated resistance element which carries the *mecA* gene, encoding the alternate penicillin-binding protein PBP2a, which confers methicillin resistance. There are currently six main types of SCC*mec*, differentiated according to their combinations of *mec* complex, containing the *mecA* gene and various portions of its regulatory genes *mecR1* and *mecI*, and *ccr* complex containing recombinases specific for the chromosomal integration and excision of the SCC*mec*. Further sub-typing is based on the presence of certain additional genes or resistance determinants within the J (so called junkyard) regions J1, J2 and J3 of the element [12]. A number of non *mecA*-encoding SCC elements, sharing some common features with various SCC*mecs*, have also been discovered in methicillin sensitive *Staphylococcus aureus* (MSSA) or coagulase-negative staphylococcal strains [13-18].

Identification of the Zurich drug clone was based on a characteristic pulsed field gel SmaI restriction pattern and the presence of a unique, previously uncharacterised SCC*mec* element which was termed SCC*mec*$_{N1}$. In addition to methicillin resistance, all drug clone isolates were shown to be resistant to trimethoprim and most were resistant to sulfamethoxazole or to ciprofloxacin. MLST typing revealed that the representative isolate of this clone, MRSA CHE482, belonged to sequence type ST45, a genotype that has been associated with epidemic MSSA and low level oxacillin resistant MRSA, which seem to have high colonization and circulation capacities [19].

All the drug clone isolates have low oxacillin resistance levels, with MICs between 0.5 and 4 μg ml^{-1}, which can make them difficult to detect by phenotypic tests. Except for the detection of the *mecA* gene, genotypic tests, which rely on identifying known features of SCC*mecs* [20,21] or SCC*mec*-chromosomal junctions [22] (X. Schneider, unpublished), have also failed to identify this clone [9].

To facilitate accurate molecular identification of this clone this manuscript presents a detailed description of the novel SCC*mec*$_{N1}$ and describes the SCC*mec* variability observed so far between different isolates.

Results and Discussion

Mapping of SCCmec$_{N1}$

The size of SCC*mec*$_{N1}$ in CHE482 was estimated to be 45.7-kb, based on a series of overlapping long range PCR products amplified with primers shown in Figure 1 and listed in Table 1. This is larger than the community-associated SCC*mec* type IV (21–25-kb), type V (27.6-kb) and type VI (approximately 22-kb) elements, falling within the range of the classical hospital associated SCC*mec* types I-III which range in size from 34–67 kb [23]. Loci of interest within SCC*mec*$_{N1}$ were then further mapped and sequenced.

mec *complex typing*

SCC*mec* typing [20] results suggested that the *mec* complex did not contain *mecI* and PCR using primers spanning IS*1272* and Δ*mecR1* and sequencing over the gene junction then confirmed the presence of a class B *mec* complex (*mecA*-Δ*mecR1*-IS*1272*).

ccr *typing*

No *ccr* complex could be detected using the multiplex PCR for *ccr* types 1 to 3 as described by Ito et al. [21], however with *ccr* type 4-specific primers C1 and C2, a weak amplificate was produced [9]. Further sequence analysis revealed that this SCC*mec* contained two complete *ccrAB* loci which are both similar to *ccrAB4* from strain HDE288, a pediatric clone isolated in Portugal carrying a type VI SCC*mec* element [24,25]. Therefore specific primers to identify the drug clone *ccrAB4-1/-2* genes were designed (primers 27 and 28). One of the loci, *ccrAB4-1*, was located at the usual *ccrAB* position downstream of the *mec* complex at the border of the J1 region. The other recombinase complex, *ccrAB4-2* was located within the J3 region (Figure 1). Sequence alignments of *ccrAB4-1*$_{CHE482}$, *ccrAB4-2*$_{CHE482}$, *ccrAB4*$_{HDE288}$ and *ccrAB4*$_{ATCC12228}$ genes showed that all four loci were different, with *ccrA4* genes sharing between 85.2% and 89.4% similarity with each other and *ccrB4* sequences sharing between 94.3% and 92.9% similarity. Nucleotide sequence similarities of these four *ccrA4* genes and *ccrA* genes from complex types 1–3, and of the four *ccrB4* genes with *ccrB* genes from complexes 1–3, are shown by phylogenetic tree (Figure 2). For these alignments the sequence of *ccrB4*$_{HDE288}$ was adjusted because the database sequence is truncated as the result of an adenine deletion at nt position 1325; leaving it 99-aa shorter than *ccrB4-1*$_{CHE482}$ and 100-aa shorter than *ccrB4-2*$_{CHE482}$.

BMC Microbiology 2007, **7**:62 http://www.biomedcentral.com/1471-2180/7/62

Table 1: Oligonucleotide primers used in this study

Primer name	Nucleotide sequence (5'-3')	Reference
Mapping and sequencing		
1	CATACACCAAGATAGACATC	This study
2	ACAACGCAGTAACTATGCAC	This study
3	GTTTATCTTCATAGACTAAC	This study
4	TTCGATGTACAATGACAGTC	IS431R, this study
5	AAGGATGTTATCACTGTAGC	IS431F, this study
6	ATGTCCCAAGCTCCATTTTG	HVR P1 F [42]
7	ACGTGTTAAGTATATTGCAC	This study
8	AAGTAGTAGCTCAACGAGCT	This study
9	CAGACAATCACATCTAACAC	This study
10	TGTTGATTGACAGTAAGGAC	This study
11	GAGTACTATAGCGTATGATGT	fusR, this study
12	ACAAACGATATGAATTCCCA	fusF, this study
13	GTTTATCTTCATAGACTAAC	This study
14	CTAATATGTTGGCGCTGATAT	This study
15	CTACACTACTATTCTTTCAC	This study
16	ATAATTACGACAATGACTGT	This study
17	CGACAATAGGATCTAAAGAT	This study
Gene detection		
18	TCCAGATTACAACTTCACCAGG	MECAP4 [20]
19	CCACTTCATATCTTGTAACG	MECAP7 [20]
20	AATAGACGTAACGTCGTACT	dfrAF, this study
21	AAGAATGTATGCGGTATAGT	dfrAR, this study
Cloning		
22	ATTAGGATCCCTAGCTGATTTAATCGTTGAAG	This study
23	ATTATCTAGATAGTAAGATATAATGTTTGGG	This study
24	ATTAGGATCCGATTGATAGTATTGCAATCA	This study
25	ATTAGGATCCGTATAGGAGTGAATGAAATGG	This study
26	ATTAGGATCCATTGTGCTTGCACAATCCTT	This study
ccrAB4-11-2$_{CHE482}$		
27	CAAATGATTGAAACAGAGGT	This study
28	CACGTTTTCTACAATAACGT	This study

By adding back this adenine we could compare the whole length sequences.

The phylogenetic trees show that *ccrAB4-1*$_{CHE482}$, *ccrAB4-2*$_{CHE482}$, *ccrAB4*$_{HDE288}$ and *ccrAB4*$_{ATCC12228}$ (*S. epidermidis*) form a distinct *ccrAB4* cluster. The presence of two complete *ccrAB4* loci in the CHE482 SCC*mec* indicated that SCC*mec*$_{N1}$ had been composed from at least two different complete or partial SCC elements. Other such mosaic or composite SCC elements have been described previously [13,26,27], however this is the first SCC*mec* found to contain two copies of the same *ccr* complex.

Due to the presence of both a class B *mec* complex and *ccrAB4*, the CHE482 SCC*mec* would be most closely related to SCC*mec* type VI. However, due to a number of unique features, including the presence of a second *ccr* locus and additional antibiotic resistance determinants, it appears to be a distinct subtype of this group that we are provisionally calling SCC*mec*$_{N1}$.

Additional resistance determinants

In addition to *mecA*, the resistance genes *dfrA* and *fusB1*, encoding trimethoprim and fusidic acid resistance, respectively, were also found on SCC*mec*$_{N1}$. The *dfrA* gene shared 100% nucleotide identity with *dfrA* from pSK1 (X13290.1), which confers high level trimethoprim resistance [28]. *dfrA* is carried on Tn*4003*, a generally plasmid-encoded composite transposon with the genetic organisation IS*431-rep*-IS*431-orf140-dfrA-thyE*-IS*431* [28]. We speculate that Tn*4003* had jumped into the SCC*mec*-associated IS*431*, hypothesised to be a hotspot for the integration of resistance determinants [29,30] (Figure 1). However, it had lost the *rep* gene (replication protein) and one of its flanking IS*431* elements, leaving Tn*4003* truncated (IS*431-orf140-dfrA-thyE*-IS*431*).

The *fusB1* gene, found within the J1 region of SCC*mec*$_{N1}$, was identical to the hypothetical fusidic acid resistance gene SAS0043 from the methicillin-susceptible strain MSSA476 [14], located on the 22.8-kb SCC-like element

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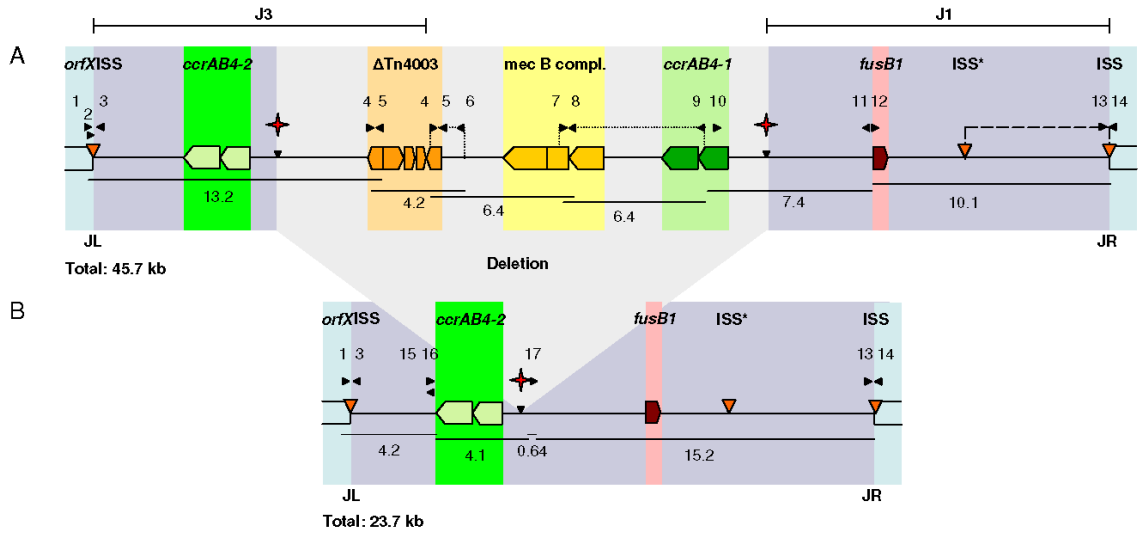

Figure 1
Schematic organisation of the SCC*mec*$_{N1}$ of strain CHE482. Primers are indicated by black arrows. Regions coloured in blue represent the core chromosome. Red stars indicate the excision sites of the partially cured strain CHE482Δ. Orange arrows indicate the ISS sites and the internal ISS*. The *ccrAB4-1* and *ccrAB4-2* complexes are highlighted in light and dark green, respectively. The truncated Tn*4003* in orange comprises IS*431*-*orf140*-*dfrA*-*thyE*-IS*431*. The class B *mec* complex in yellow includes *mecA*-Δ*mecR1*-IS*1272*. The fusidic acid resistance gene *fusB1* is shown in pink. Dotted lines indicate regions of variability. The region absent in ZH4 and ZH43 is indicated by a dashed line. JL: junction left; JR: junction right. Positions of SCC*mec* regions J1 and J3 are shown. A: Entire SCC*mec*$_{N1}$ in CHE482. B: Partially cured variant CHE482Δ.

SCC$_{476}$, in MSSA476. The SCC$_{476}$ *ccrAB* genes, however, are most similar to the type 1 *ccr* complex from *S. hominis*. The *fusB1* gene in CHE482 conferred only low level fusidic acid resistance of 6 μg ml^{-1}.

SCCmec boundaries

The boundaries of the SCC*mec* element were sequenced using the primers 2, 3, 13 and 14 and compared to reference sequences of SCC$_{476}$ from MSSA476 and SCC*mec* type II from N315 (Figure 3). SCC*mec*$_{N1}$ had integrated at the same position within the *attB*$_{SCC}$ sequence at the 3' end of *orfX* as all previously described SCC*mec* and SCC elements. The ends of SCC*mec*$_{N1}$ contained the characteristic direct and degenerate-inverted repeats found at the ends of SCC*mec* types I-IV and SCC$_{476}$. Integration site sequences (ISS) with the consensus sequence 5'-(GANGCNTATCATAANTN)-3 [23] were present at both boundaries. A third ISS sequence (ISS*) was also identified about 6.4-kb upstream from the right end junction.

Drug clone variability

Analysis of PFGE profiles of all drug clone isolates characterised by Qi et al. in 2003 [9] revealed that there were small variations in size in the 208-kb SmaI band containing *mecA*. Therefore the SCC*mec* of CHE482 and a selection of three other drug clone strains (ZH4, ZH43 and ZH81, Table 2) were cured using plasmid pSR3-1. The SmaI band carrying SCC*mec* was slightly larger in CHE482 and ZH81 than in ZH4 and ZH43 before curing, whereas after curing the resulting patterns were identical in all four strains (Figure 4A). This indicated that there was variability, presumably within the SCC*mec*.

SCCmec$_{N1}$ variation

Using the long range overlapping PCR products, variations in the SCC*mec* elements of strains CHE482, ZH4, ZH43 and ZH81, were compared. Fragment sizes between primers 6 and 4 varied by 100 to 200 bp, and between primers 7 and 9 from 1000 to 1500 bp. The variation in the hypervariable region between the *mec* complex and ΔTn*4003* (primers 6 and 4) could be due to different numbers of direct repeats units (*dru*) [31] as found in SCC*mec* V$_T$ compared to the WIS SCC*mec* V [32]. Amplification between *fusB1* and the right SCC*mec* junction produced a 6.4-kb larger end fragment from CHE482/ZH81 than from ZH4/ZH43.

BMC Microbiology 2007, **7**:62 http://www.biomedcentral.com/1471-2180/7/62

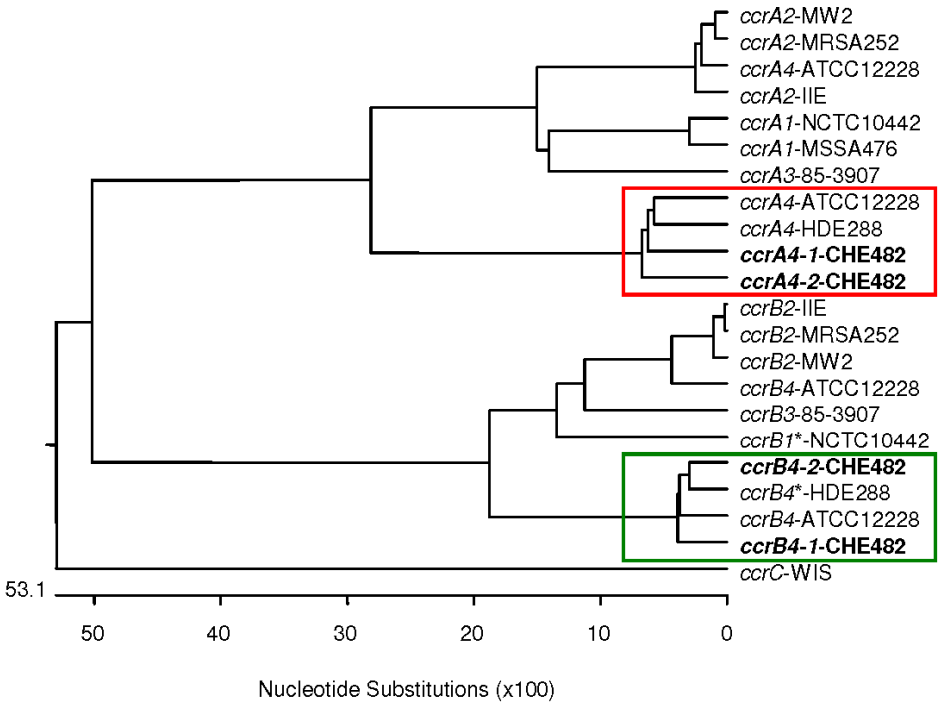

Figure 2
Phylogenetic relatedness of selected *ccrA* and *ccrB* nucleotide sequences. The following genes were used: *ccrA1* and *ccrB1** from strain NCTC10442 [DDBJ:AB033763]; *ccrA2* and *ccrB2* from MRSA252 [EMBL:BX571856], MW2 [NCBI:NC_003923], SCC*mec* IIE [EMBL:AJ810120]; *ccrA3* and *ccrB3* of 85–3907 [DDBJ:AB047088]; *ccrA4* and *ccrB4** from HDE288 [GenBank:AF411935]; *ccrA4* and *ccrB4* of ATCC12228 [GeneBank:AE015929]; *ccrC* in WIS [DDBJ:AB121219]. The *ccrB* genes from NCTC10442 (*ccrB1**) and HDE288 (*ccrB4**) are truncated; for comparison we have reconstituted them by adding an adenine at the site of the frameshifts. The evolutionary relationships are shown by the length of the branches and the scale of the tree indicates the number of nucleotide substitutions per 100 bases. Alignment was done using ClustalW and tree constructed with Multalign, Lasergene 6.0. Type 4 *ccrA* genes are framed in red and and type 4 *ccrB* genes in green.

Sequencing in from the right junctions showed that the ends of CHE482/ZH81 were identical to each other with no significant similarity to any database sequences (data not shown) but they were different to those of ZH4/ZH43 (Figure 3). The latter sequences of ZH4/ZH43 were identical to the end of SCC_{476}. In contrast, the left end chromosome-SCC*mec* junction sequences were identical in all drug clones analysed.

Antibiotic resistance variability

SCC*mec* variability also appeared to correlate with other strain differences. Strains ZH4 and ZH43 which had identical SCC*mecs*, were also both ciprofloxacin resistant; meanwhile CHE482 and ZH81, which share identical SCC*mecs*, were ciprofloxacin susceptible (Table 2). There was also variation in fusidic acid resistance levels. Most strains had relatively low fusidic acid resistance. ZH43, however, was highly resistant and resistance was not lost upon curing (Table 3), therefore resistance in this strain was probably additionally caused by a mutation in the chromosomal elongation factor G, EF-G (*fusA*) [33]. Since both ciprofloxacin and sulfamethoxazole resistance are chromosomal, the SCC*mec* variants found in the Zurich drug clones are very likely associated with different, closely related genetic backgrounds.

ccr activity

CHE482 was cured using either pME21 (*ccrAB4-1*) or pME22 (*ccrAB4-2*). Resulting isolates were screened for methicillin, trimethoprim and fusidic acid susceptibility and for amplification of a PCR product spanning the former SCC*mec*-chromosomal junctions (primers 1 and 14, Figure 1). Both *ccrAB4-1* and *ccrAB4-2* were functional and able to excise SCC*mec* even though their *ccrA* and *ccrB*

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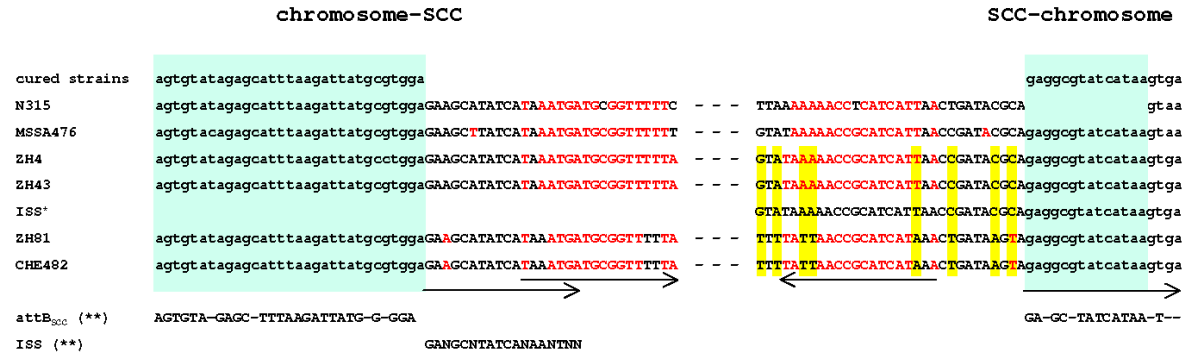

Figure 3
Chromosome-SCC*mec* junction sequences. The borders of strain CHE482 were aligned with the sequences of the three drug clones, ZH4, ZH43, ZH81, the cured drug clone, strain N315 (type II SCCmec), MSSA476 (SCC_{476})and ISS*. Light green boxes indicate the *orfX* region and its integration sequence site, ISS. Yellow shading highlights differences between the SCC*mec* ends. Direct and indirect repeats are indicated with arrows. The red letters represent the similarity of the inverted repeats. $attB_{SCC}$** and ISS** consensus sequences were taken from Ito et al. [23].

amino acid sequences differed by 11.3% and 4.6%, respectively. This is consistent with the finding that several different *ccrAB* loci from type IV SCC*mec*s were all shown to be active, despite varying up to 3.7% in their amino acid sequences [34].

Excision variants

CHE482 was cured using pSR3-1 [35], the resulting strain ME21 was sensitive to oxacillin, fusidic acid and trimethoprim (Figure 1, Table 3). During curing experiments with pME21 and pME22 we discovered that there were also many strains that had not been completely cured. One set of cured CHE482 variants maintained a fragment of 6.4-kb, and sequencing confirmed that this fragment was the portion between the ISS at the right junction and ISS*. This indicated that excision of the main SCC*mec* fragment containing all three resistance determinants had occurred through recombination between the ISS at the left junction and ISS*.

ccrAB4-1 and its predicted promoter were also cloned into the *E. coli-S. aureus* shuttle vector pAW17 to produce the recombinant plasmid pME15. Attempts to cure CHE482 of its SCC*mec* element using pME15 resulted in another partially cured set of variants which had maintained fusidic acid resistance but lost oxacillin and trimethoprim resistance (CHE482Δ, Figure 1B). Analysis of these strains by PFGE, showed that the SCC*mec*-containing band had become smaller but not to the same extent as the completely cured strain ME21 (Figure 4B). These results indicated that only a portion of the SCC*mec*, containing *mecA* and *dfrA*, had been lost. PCR mapping identified the location of the missing portion and sequencing over the excision sites revealed that excision was likely to be mediated by homologous recombination across regions of high nucleotide sequence similarity surrounding the two *ccr* loci, as no additional ISS sequences were found. It appeared that recombination between the two *ccr* regions resulted in the deletion of a 22-kb fragment containing *ccrAB4-1*, the class B *mec* complex and ΔTn*4003*. This recombination left an SCC-like element of 23.7-kb, which contained one *ccrAB* complex (*ccrAB4-2*) and the fusidic acid resistance determinant (Figure 1B). This truncated SCC is similar in size to the MSSA476 SCC_{476} which also contains *fusB1* and a *ccrAB* locus, although in SCC_{476} the *ccr* genes are most similar to *ccrAB* type 1.

Therefore we have identified three possible excision variants, two resulting from the presence of three ISS, as has been seen in SCC*mec* type IV strains [26], and the third variant caused by recombination between regions of high sequence similarity.

Conclusion

The general structure of $SCCmec_{N1}$ (*ccrAB4-2*, *dfrA*, class B *mec* complex, *ccrAB4-1*, *fusB1*) was distinctly different from already published SCC*mec* types. Several regions of variability were found between different clinical drug clone isolates, especially in the right-end region where the presence or absence of a DNA fragment framed by ISS sequences was detected. Nevertheless this clone can now be identified by its resistance profile and its combination of class B *mec* complex and *ccrAB4* complex sequences, thus allowing easier epidemiological identification.

BMC Microbiology 2007, **7**:62 http://www.biomedcentral.com/1471-2180/7/62

Table 2: Strains and plasmids

Strain	Relevant genotype	Phenotype	Origin, Reference
S. aureus			
CHE482	CC45, ST45, SCC*mec*$_{NI}$ (*dfrA, fusB1*), *blaZ*	Mc^r, Tm^r, Fa^r, Sx^R	IMM collection, University Zurich
ME21	CHE482ΔSCC*mec*$_{NI}$, *blaZ*	Mc^s, Tm^s, Fa^s, Sx^R	this study
CHE482Δ	CHE482Δ'SCC*mec*$_{NI}$ (*fusB1*), *blaZ*	Mc^s, Tm^s, Fa^r, Sx^R	this study
ZH81	SCC*mec*$_{NI}$ (*dfrA, fusB1*), *blaZ*	Mc^r, Tm^r, Fa^r	[9]
ME141	ZH81ΔSCC*mec*$_{NI}$, *blaZ*	Mc^s, Tm^s, Fa^s	this study
ZH4	SCC*mec*$_{NI}$(*dfrA, fusB1*), *blaZ*	Mc^r, Tm^r, Cp^r, Fa^r, Sx^R	[9]
ME135	ZH4ΔSCC*mec*$_{NI}$, *blaZ*	Mc^s, Tm^s, Cp^r, Fa^s, Sx^R	this study
ZH43	SCC*mec*$_{NI}$ (*dfrA, fusB1*), *blaZ, fusA*	Mc^r, Tm^r, Cp^r, Fa^r	[9]
ME138	ZH43ΔSCC*mec*$_{NI}$, *blaZ, fusA*	Mc^s, Tm^s, Cp^r Fa^r	this study
HDE288	Pediatric clone, type 4 *ccr* complex	Mc^r	[24, 43]
E. coli			
DH5α	restriction-negative strain for cloning		Invitrogen
Plasmids			
pYT3	ori(ts), *S. aureus, tetL*	Tc^r	[35]
pSR3-1	ori(ts) *S. aureus, ccrAB2* genes, *tetL*	Tc^r	[35]
pAW17	*S. aureus-E. coli* shuttle vector, *aac-aph*	Km^r	[44]
pME15	pAW17 *ccrAB4-1*$_{CHE482}$	Km^r	This study
pME21	pYT3 *ccrAB4-1*$_{CHE482}$	Tc^r	This study
pME22	pYT3 *ccrAB4-2*$_{CHE482}$	Tc^r	This study

Abbreviations: Ap, ampicillin; CC, clonal complex; Cp, ciprofloxacin; Fa, fusidic acid; Gm, gentamicin; Km, kanamycin; Mc, methicillin; Ox, oxacillin; ST, sequence type; Sx, sulfomethoxazole; Tc, tetracycline; Tm, trimethoprim; ts, temperature sensitive.

Materials and methods

Bacterial strains and growth conditions

Bacterial strains and plasmids are listed in Table 2. The four clinical MRSA isolates CHE482, ZH4, ZH43 and ZH81 were clones associated with intravenous drug users in the Zurich area. Apart from the type strain CHE482, strains were selected from the epidemiological study in 2003 based on their PFGE patterns and resistance profiles (Table 2) [9]. Growth was at 37°C in Luria Bertani broth (Difco Laboratories, Detroit, MI, USA). Strains harbouring the temperature-sensitive plasmids pME21 or pME22 were propagated at 30°C in the presence of 10 μg ml^{-1} tetracycline and those with plasmid pME15 were grown at 37°C in the presence of 50 μg ml^{-1} kanamycin.

Susceptibility testing

The minimal inhibitory concentrations (MIC) of antibiotics were determined by Etest on Mueller-Hinton agar plates (Difco Laboratories, Detroit, MI, USA) according to the manufacturer's instructions (AB Biodisk, Solna, Sweden). Disc diffusion of oxacillin, cefoxitin, fusidic acid and trimethoprim/sulfamethoxazole were done according to CLSI [36] on Mueller Hinton agar plates. Penicillinase production of cefoxitin-induced cells was assayed by nitrocefin hydrolysis and PBP2a production by the MRSA screen agglutination test from Denka Seiken (Tokyo, Japan) [37].

SCCmec typing

SCC*mec* types I through IV, *ccr* types 1 to 3, and *ccr* type 4 from the pediatric clone HDE288, were identified by PCR as described by [20,21], and [38], respectively. A specific PCR was established to identify the drug clone *ccrAB4-1/-2*$_{CHE482}$ using primer pair 27 and 28 (Table 1).

Localization of dfrA *and* mecA

SmaI digested chromosomal DNA was separated by pulsed field gel electrophoresis, PFGE [39] and hybridised sequentially [40] with a *mecA* (primer pair 18 and 19) and a *dfrA* probe (primer pair 20 and 21) (Table 1).

Cloning of the ccrAB *genes of CHE482*

Each of the two *ccrAB* complexes identified in strain CHE482, including their own promoter, were cloned into the BamHI site of the temperature-sensitive plasmid pYT3, using primers 22 and 24 for *ccrAB4-1*$_{CHE482}$ and primers 25 and 26 for *ccrAB4-2*$_{CHE482}$. The resulting plasmids pME21 and pME22, respectively, were electroporated into RN4220 and then transduced by Φ80α into the MRSA clinical isolates to be cured of SCC*mec*. The *ccrAB4-1*$_{CHE482}$ was also cloned into the *E. coli-S. aureus* shuttle vector pAW17, using the primers 22 and 23 (Table 1). The resulting plasmid pME15 was then electroporated into RN4220 and transduced into the MRSA to be cured of SCC*mec*.

Curing of SCCmec

Curing of SCC*mec* was done by the method of Katayama [35] using either the temperature-sensitive plasmid pSR3-1 containing *ccrAB* type 2 recombinase genes, or plasmids pME21 (*ccrAB4-1*) or pME22 (*ccrAB4-2*). Curing with plasmid pME15 (*ccrAB4-1*) was done by transducing the

BMC Microbiology 2007, 7:62 http://www.biomedcentral.com/1471-2180/7/62

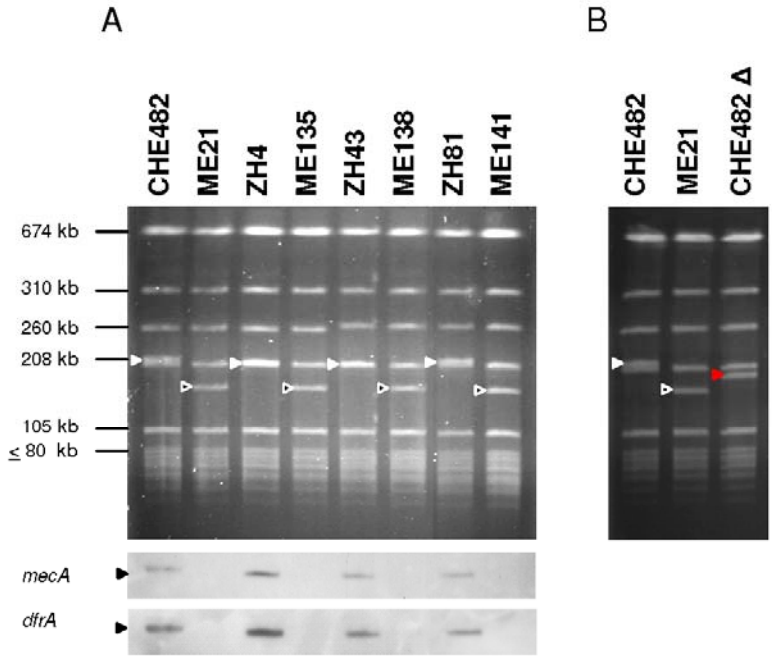

Figure 4
PFGE restriction analysis. A: SmaI restriction patterns of four drug clone isolates CHE482, ZH4, ZH43, ZH81 and their corresponding cured strains ME21, ME135, ME138, ME141. The SmaI fragments carrying SCC*mec* are indicated by filled triangles, and the corresponding fragments of the cured strain are indicated by open triangles. Southern hybridisations using a *mecA* and a *dfrA* probe are shown below. B: PFGE patterns of the drug clone CHE482 (filled triangle), its cured strain ME21 (open triangle) and the partially cured strain CHE482Δ (red triangle).

plasmid into the clinical MRSA isolates, selecting for kanamycin resistant transductants at 37°C, which were then pooled and plated on LB agar containing kanamycin, grown overnight, and screened by replica plating for loss of oxacillin resistance on 1 μg ml^{-1} oxacillin.

Sequence analysis

Sequencing was performed with an ABI PRISM BigDye Terminator Cycle sequencing reaction kit (US Biochemicals, Cleveland, Ohio) and an ABI Prism 310 genetic analyzer (Applied Biosystems, Foster City, California).

Table 3: Antibiotic minimal inhibitory concentration [μg ml^{-1}]

strain	OX	FX	TR	FA
CHE482	1.5	8	> 32	6
ME21	0.38	4	0.38	0.125
CHE482Δ	0.38	3	0.25	6
ZH4	1.5	6	> 32	6
ME135	0.50	3	0.38	0.125
ZH43	1.5	8	> 32	> 256
ME138	0.50	3	0.50	> 256
ZH81	0.75	6	> 32	6
ME141	0.38	3	0.75	0.19
HDE288	1.5	16	0.50	NA

Abbreviations: FA, fusidic acid; FX, cefoxitin; NA, not analysed; OX, oxacillin; TR, trimethoprim.

BMC Microbiology 2007, **7**:62 http://www.biomedcentral.com/1471-2180/7/62

Sequence assembly was accomplished using the DNAStar sequence analysis package (Lasergene 6.0).

Sequencing of the SCC*mec*-chromosome junctions of four drug clones was done by direct chromosomal sequencing [41] of the original and cured clones using primer 2. This nucleotide sequence provided the template for the design of primers 14, 13 and 3 (Table 1) over the chromosome-SCC*mec* junctions.

Mapping of SCCmec

To estimate the size of the CHE482 SCC*mec*, long range PCR amplification of six main fragments was performed using the polymerase TaKaRa Ex Taq (TAKARA BIO INC., Shiga, Japan). PCR was done as described by the manufacturer's recommendation. Primer pairs utilised were: 1 and 5; 4 and 6; 4 and 8; 7 and 9; 10 and 11 and 12 and 14 (Table 1). Amplified PCR fragments were run against molecular weight markers (Marker II, Fermentas International, Ontario, CA; 1 kb+, Invitrogen, Carlsbad, CA) on a 0.5% agarose gel.

Nucleotide sequence accession number

The sequences of *ccrAB4-1*$_{CHE482}$ and *ccrAB4-2*$_{CHE482}$ have been deposited in the GenBank (NCBI) database under accession number [GenBank: EF126185] and [GenBank:EF126186] respectively.

Authors' contributions

ME carried out the molecular genetic and microbiological studies and drafted the manuscript. BB conceived of the study, participated in its design and helped to draft the manuscript. NM participated in the design and coordination of the study and helped to draft the manuscript. All authors read and approved the final manuscript.

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3.2 Project 2: Impact of mecA promoter mutations on mecA expression and β-lactam resistance levels

Abstract

The reason for the extremely low-level oxacillin resistance in a so called “drug clone”, a methicillin-resistant *Staphylococcus aureus* circulating among injection drug users in Zurich, Switzerland, could be traced back to the *mecA* promoter sequence and particularly to the strain's genetic background. Sequencing of its mec complex identified a point mutation (TATA**C**T to TATA**T**T), creating a perfect palindrome in the -10 region of the *mecA* promoter/operator region containing the binding sites for the *mecA* repressors MecI and BlaI. Two strains with vastly different β-lactam resistance phenotypes, the low-level resistant drug clone type strain CHE482 and the highly homogenously resistant strain COLn, were cured of their SCC*mec* elements and subsequently transformed with plasmids containing *mecA* under the control of either the wildtype or mutant promoter. Expression studies showed that this mutation had significant effects on both *mecA* transcription and corresponding PBP2a production, but only small effects on β-lactam resistance levels within a given genetic background. A further mutation in the *mecA* ribosomal binding site (GGAG**G** to GGAG**T**), common to SCC*mec* type IV strains, was found to have no discernable effect on *mecA* transcription and PBP2a content, and only minimal effects on β-lactam resistance. Factors associated with the genetic backgrounds into which these differently controlled *mecA* genes were introduced, had a much higher impact on β-lactam resistance levels than the rates of *mecA* transcription. The tight repression of *mecA* expression in this drug clone in the absence of β-lactams, could contribute to the apparent fitness of this fast growing strain.

Introduction

Methicillin resistant *Staphylococcus aureus* (MRSA) can express highly variable levels of β-lactam resistance. Oxacillin minimal inhibitory concentrations (MIC) can vary from as low as 1 µg ml^{-1} up to values over 1000 µg ml^{-1}. Extremely low-level resistant MRSA strains are dangerous as they often evade phenotypic detection. While they appear phenotypically susceptible, these strains still carry the *mecA* gene, which encodes the β-lactam resistance protein penicillin binding protein (PBP) 2a, and express resistance heterogeneously. This means that upon β-lactam exposure they can segregate highly resistant subpopulations at frequencies well above spontaneous mutation rates, and cause β-lactam treatment failure.

β-lactam resistance relies on the presence of *mecA* and the expression of PBP2a, however, several other factors are known to influence resistance. Chromosomal mutations or specific genomic factors are required for expression of high level resistance (Murakami and Tomasz, 1989; Ryffel et al., 1994; Sieradzki et al., 2007). Resistance levels are also influenced by environmental conditions such as temperature, pH, osmolarity, divalent cations and aerobiosis (Matthews and Steward, 1984). The actual genetic basis governing low-level, heterogeneous or high homogeneous resistance is unknown.

Expression of *mecA* is inducible and can be controlled by either its cognate regulators MecI (DNA binding repressor protein) and MecR1 (sensor/signal transducer) or by the structurally and functionally similar β-lactamase regulators BlaI and BlaR1, respectively. Because of structural and functional similarity, both MecI and BlaI can bind as homodimers to the promoter/operator region of both *mecA* and *blaZ* (Gregory et al., 1997; Sharma et al., 1998). Repression of transcription by these two inhibitors is even stronger when both repressors are present (Rosato et al., 2003a). In the presence of β-lactams the MecR1/BlaR1 sensor-transducers promote the cleavage of their respective repressors allowing transcription of *mecA*. In the absence of both regulatory loci, *mecA* is constitutively expressed.

High conservation of the *mecA* gene sequence (Chambers, 1997), promoter region and the preferred presence of regulatory genes (*blaI/blaR1* or *mecI/mecR1*) (Rosato et al., 2003a) highlights the need for controlled expression of *mecA*. In strains such as N315, a so called pre-MRSA (Niemeyer et al., 1996; Weller, 1999) carrying both complete regulatory systems, *mecA* is repressed by both MecI and BlaI. This tight repression can prevent *mecA* transcription and lead to inhibition of β-lactam resistance and to the misinterpretation of these strains as methicillin sensitive *S. aureus* (MSSA).

The amount of PBP2a produced has been shown to have no direct correlation with resistance level, however mutations in *mecA*, *mecI*, the *mecI* ribosomal binding site, or *mecI* deletion, can lead to increased resistance (Katayama et al., 2004; Niemeyer et al., 1996; Rosato et al., 2003a), whereas interruption of *blaR1* results in repression and therefore decreased resistance (Hackbarth et al., 1994).

An MRSA strain with very low-level oxacillin resistance, with the ability to generate highly resistant subclones, is spreading among injection drug users in Zurich, Switzerland (Qi et al., 2005). Isolates of this clone having oxacillin MICs of 0.5-4 µg ml^{-1} are susceptible or just at the breakpoint of resistance for oxacillin, according to CLSI guidelines. They fall within the classification of the recently described OS-MRSA (oxacillin susceptible methicillin-resistant *S. aureus*), defined as being oxacillin sensitive but *mecA*-positive strains with MICs of 2 µg ml^{-1} or below (Hososaka et al., 2007). A recent analysis of the SCC*mec* element of the drug clone type strain CHE482 identified a composite element carrying a mecB complex (IS*1272-ΔmecR1-mecA*) (Ender et al., 2007). Further investigation of the mec complex revealed that the *mecA* promoter differed from the consensus by a 1-bp substitution in the -10 region. Here we determined the influence of this nucleotide substitution on *mecA* transcription and β-lactam resistance levels in different genetic backgrounds, using MRSA cured from their SCC*mec* element as recipients.

Materials and methods

Bacterial strains and growth conditions. All strains and plasmids used in this study are listed in Table 1. Strains were grown in Luria Bertani broth (LB, Difco) at 37°C, with shaking at 180 rpm. Media were supplemented with 10 µg ml^{-1} tetracycline for strains harbouring the plasmids pME1, pME2 or pME3 and with 0.8 µg ml^{-1} ampicillin for those containing the penicillinase plasmids pBla or pI524.

Table1. Strains and plasmids used in this study.

Strain	Relevant genotype	Relevant phenotype	Origin, Reference
Staphylococcus aureus			
CHE482	CC45, ST45, SCC*mec*$_{N1}$, *blaZ* (pBla)	Mc^r, Ap^r, Fa^r, Tm^r	(Ender et al., 2007; Qi et al., 2005)
ME21	CHE482ΔSCC*mec*$_{N1}$, *blaZ* (pBla)	Mc^s, Ap^r Fa^s, Tm^s	(Ender et al., 2007)
COLn	COL derivative, SCC*mec* type I	Mc^r, Tc^s	(Katayama et al., 2004)
ME131	COLnΔSCC*mec* type I	Mc^s, Tc^s	(McCallum et al., 2006b)
ZH47	CC5, ST100, composite SCC*mec*, *aac*(6')-*aph*(2''), *blaZ*	Mc^r, Gm^r, Km^r	(Heusser et al., 2007)
Escherichia coli			
DH5α	restriction-negative strain for cloning		Invitrogen
Plasmids			
pBUS1	*S. aureus-E. coli* shuttle vector, *tetL*	Tc^r	(Rossi et al., 2003)
pME1	pBUS1-*mecA* and its promoter of CHE482	Tc^r	this study
pME2	pBUS1-*mecA* and its promoter of COLn	Tc^r	this study
pME3	pBUS1-*mecA* and its promoter of ZH47	Tc^r	this study
pBla	β-lactamase plasmid of CHE482	Ap^r	this study
pI524	β-lactamase plasmid	Ap^r	(Murphy and Novick, 1980)

Abbreviations: Ap, ampicillin; CC, clonal complex; Fa, fusidic acid; Gm, gentamicin; Km, kanamycin; Mc, methicillin; ST, sequence type; Tc, tetracycline; Tm, trimethoprim.

Curing of SCC*mec*. Strains CHE482 and COLn were cured of SCC*mec* using the method described by Katayama et al. (Katayama et al., 2000), resulting in strains ME21 and ME131, respectively.

Cloning and transformation. The *mecA* gene, together with its upstream promoter region, was amplified from strains CHE482, COLn and ZH47 using the primer pair (5'-ATTAGGATCCCCAAATCTTATGTGACATAA-3'/5'-ATTAGGATCCATCCTCAATATATGCA TATAG-3'), and subsequently cloned into the BamHI site of the *E. coli-S. aureus* shuttle vector pBUS1. The resulting plasmids pME1, pME2 and pME3, as well as pBla, the native penicillinase plasmid of CHE482, and pI524 a penicillinase plasmid described in Murphy et al. (Murphy and Novick, 1980) were electroporated directly into ME21 and ME131. Direct electoporation was done as described by Katayama et al. (Katayama et al., 2003), whereby cells were mixed with 500 ng of plasmid DNA, incubated for 10 min on ice and electroporated using following settings: 25 µF, 2.0 kV and 100 Ω.

Sequence analysis. The plasmids pME1, pME2 and pME3 were sequenced with an ABI Prism 310 genetic analyzer (Applied Biosystems) using the ABI PRISM BigDye Terminator Cycle sequencing reaction kit (U.S. Biochemicals). Thermal stability of inverted repeats (ΔG) was calculated using the DINAMelt Server (Markham and Zuker, 2005).

Growth curves. Strains were grown overnight in LB (Difco) in the presence of appropriate antibiotics, diluted 1:200 and grown for another 3 h. This preculture was used to inoculate 50 ml of fresh prewarmed broth at a 1:1,000 dilution. Measurements were taken every 30 min at OD_{600nm} and the minimal doubling time calculated.

Susceptibility testing. The MIC of antibiotics was determined by Etest (AB Biodisk) on Mueller-Hinton plates (Difco) swabbed with an inoculum of 0.5 McFarland and incubated at 35°C for 24 h, according to the manufacturer's instruction (AB Biodisk). Qualitative comparisons of cefoxitin resistance levels were obtained by swabbing 0.5 McFarland suspensions of strains across agar plates containing increasing concentration gradients of cefoxitin. Gradients of cefoxitin used were 0-8 µg ml^{-1} and 0-500 µg ml^{-1}. The production of penicillinase was confirmed by nitrocefin hydrolysis of cefoxitin induced cells.

Northern blot analysis. A preculture, as described for the growth curves, was used to inoculate 150 ml LB broth at a dilution factor of 1:1,000. Cells were grown at 37°C to OD_{600nm} 1.0 then induced with cefoxitin 4 µg ml^{-1} and sampled at time point 0' before induction and at 5', 10', 20' and 30' after induction. Cells were pelleted at 4°C for 3 min then snap-frozen in liquid nitrogen. Total RNA was extracted as described in Cheung et al. (Cheung et al., 1994). RNA samples (8 µg) were separated in a 1.5% agarose gel containing 20 mM guanidine thiocyanate in 1x TBE (Goda and Minton, 1995). RNA transfer and detection were performed as described previously (McCallum et al., 2006a). Primers used to amplify DIG labelled

probes for *mecA* were, mecAP4/mecAP7 (Oliveira and de Lencastre, 2002) and for *blaZ*, primer pair (5'-TGTGACTTACTTTCAACTGT-3' / 5'-TTACGATCCTGAATGTTTAT-3').

Western blot analysis. Extraction of cell wall proteins, from cells harvested before (0') and after induction with cefoxitin 4 µg ml^{-1} (10', 20'), was done as described in Katayama et al. (Katayama et al., 2004) with minor modifications. Additionally, a sonification step (3 x 20 sec) was introduced after lysing the cells with lysostaphin (100 µg ml^{-1}) and adding DNase (20 µg ml^{-1}) and RNase (10 µg ml^{-1}). Ultracentrifugation was performed at 40,000 g for 40 min. After washing with 50 mM phosphate buffer pH7, the pellet was resuspended in 100 µl phosphate buffer.

Protein concentrations were measured by Bradford assay (BioRad) (Bradford, 1976). After separation of 10 µg of protein extract on a 7.5% SDS-polyacrylamide gel, the proteins were blotted onto a nitrocellulose membrane (Hybond) and stained with Ponceau to confirm equal protein loading. For detection, membranes were blocked for 1 h with 5% milk powder in 1 x low salt buffer (77.5 mM NaCl, 4.5 mM Tris, 0.05% Tween20, pH 7.4), washed with 0.5% milk powder in low salt buffer containing 40 µg ml^{-1} human IgG (Calbiochem) then PBP2a antibody (1:20,000, Denka Seiken) was added after 30 min and was incubated for another hour. After 3 x 5 min washing with 1 x low salt buffer the secondary antibody, an anti-mouse IgG HRP conjugate (1:2,500) in 1 x low salt buffer containing 0.5% milk powder, was added and incubated for 1 h. Membranes were washed again for 3 x 5 min in 1 x low salt buffer. Detection was performed using SuperSignal West Pico Chemiluminescent Substrate (Pierce).

Results

Sequence of CHE482 *mecA* promoter. The low level oxacillin resistant drug clone CHE482 carries a type B mec complex consisting of the *mecA* gene with a truncated, non-functional regulatory locus. Nucleotide sequence analysis of the *mecA* gene and promoter region revealed one single base pair mutation within the *mecA* promoter consensus sequence. This C to T substitution localized in the -10 promoter region, 33-nt upstream of the *mecA* translational start site, resulted in the formation of a perfect 30-bp palindrome encompassing the binding sites for MecI/BlaI surrounding the -10 box (Figure 1). The substitution increased the stability of the inverted repeat to a ΔG of -10.8 kcal mol^{-1}, compared to the consensus promoter represented here by COLn, which had a ΔG of -8.8 kcal mol^{-1}. Three other randomly chosen drug clone isolates with MICs between 0.5 and 3 μg ml^{-1} showed the same nucleotide substitution, indicating that the mutation to a perfect palindrome was apparently a common feature of this clonal lineage.

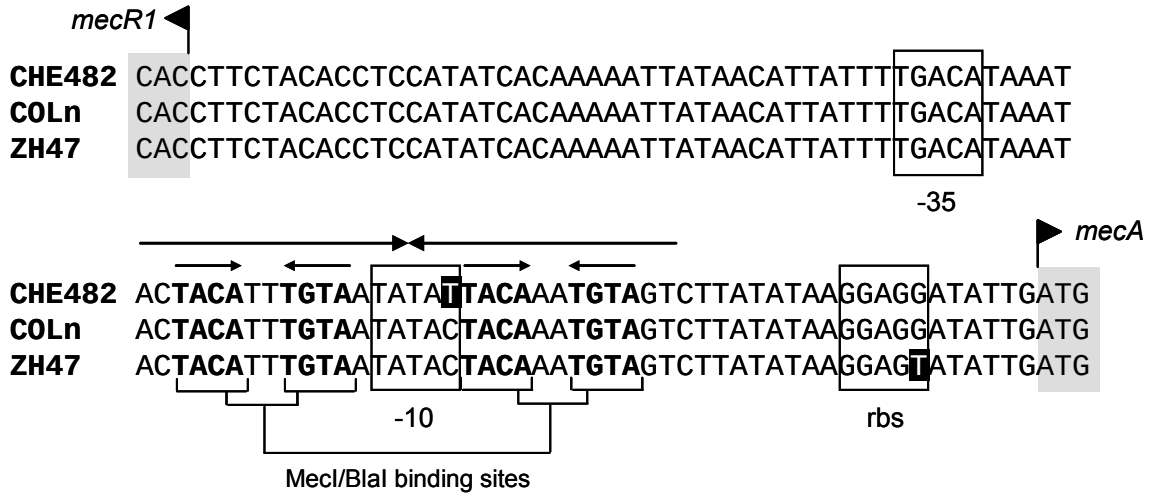

Figure 1.

mecA promoter sequence of CHE482 compared to that of COLn (consensus promoter sequence) and ZH47. Direct and indirect repeats are indicated by arrows. The -10, -35 regions and the ribosomal binding site (rbs) are framed. The start codons of *mecA* and *mecR1* are highlighted in grey and the direction of transcription indicated by arrows. Binding sequences of MecI/BlaI are shown in bold. Point mutations in CHE482 and ZH47 compared to COLn (designated as the consensus) are highlighted in black.

Influence of *mecA* promoter variants on *mecA* expression and PBP2a production. The position of the nucleotide substitution in the *mecA* promoter region suggested that it may influence induction of *mecA* expression and hence be a contributing factor to the low-level resistance of CHE482. To compare the effect of the two different promoter sequences on

mecA transcription, plasmids pME1, containing the *mecA* gene and promoter from CHE482; and pME2, containing the *mecA* gene with a consensus sequence promoter from the highly homogenously resistant MRSA strain COLn, were constructed. To assess *mecA* transcription and PBP2a production in both the CHE482 and COLn genetic backgrounds, CHE482 and COLn were first cured of their SCC*mec* elements, creating MSSA strains ME21 and ME131, respectively. Plasmids pME1, pME2, and the empty vector, as a control, were then electroporated directly into the cured strains ME21 and ME131 (Figure 2).

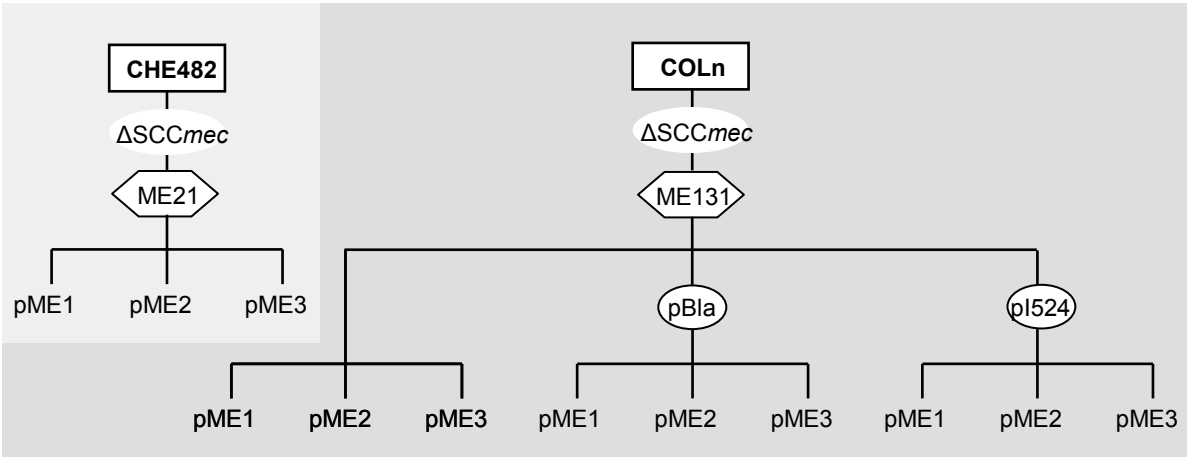

Figure 2.

Scheme of strain construction. Original MRSA strains are framed by rectangles, the cured strains beneath are framed by hexagons. Plasmids pME1, pME1, pME3 were introduced into the cured strains. In the case of COLn, two different penicillinase plasmids (framed by ovals) were transformed into the cured strain ME131. The three plasmids pME1, pME1, pME3 were introduced into each of the three variants, ME131, ME131pBla and ME131pI524.

Northern blots were used to compare *mecA* transcription from pME1 and pME2 in both the CHE482- and COLn-derived strains ME21 and ME131, respectively, before and after induction with a sub-inhibitory concentration of cefoxitin (Figure 3A). In ME21, which contains an inducible β-lactamase, *mecA* transcription is subject to repression and β-lactam induction over the BlaI/BlaR1 regulatory system. Consequently, both plasmids showed increased *mecA* transcription upon cefoxitin induction in ME21, although, the levels of transcription differed markedly. In pME1, transcription was strongly repressed before induction and steadily increased over the 30 min period post induction, in a similar manner to *blaZ* induction. In contrast, *mecA* transcription levels from pME2 were already relatively high before induction and only showed a small increase after 20-30 min of induction. Transcription of *mecA* from pME1 and pME2 also differed in ME131, which contains neither MecR1/MecI nor BlaR1/BlaI regulatory elements. While *mecA* was constitutively transcribed from both plasmids, transcription was significantly higher from pME2. Western blots, quantifying PBP2a

production, showed that in most cases there was a correlation between *mecA* transcription and PBP2a expression levels (Figure 3A). Amounts of PBP2a produced did however appear to be higher in the ME131 than in the ME21 strain background, as seen by higher overall PBP2a signals in both ME131 strains and a lack of PBP2a induction above the basal level in strain ME21 pME2.

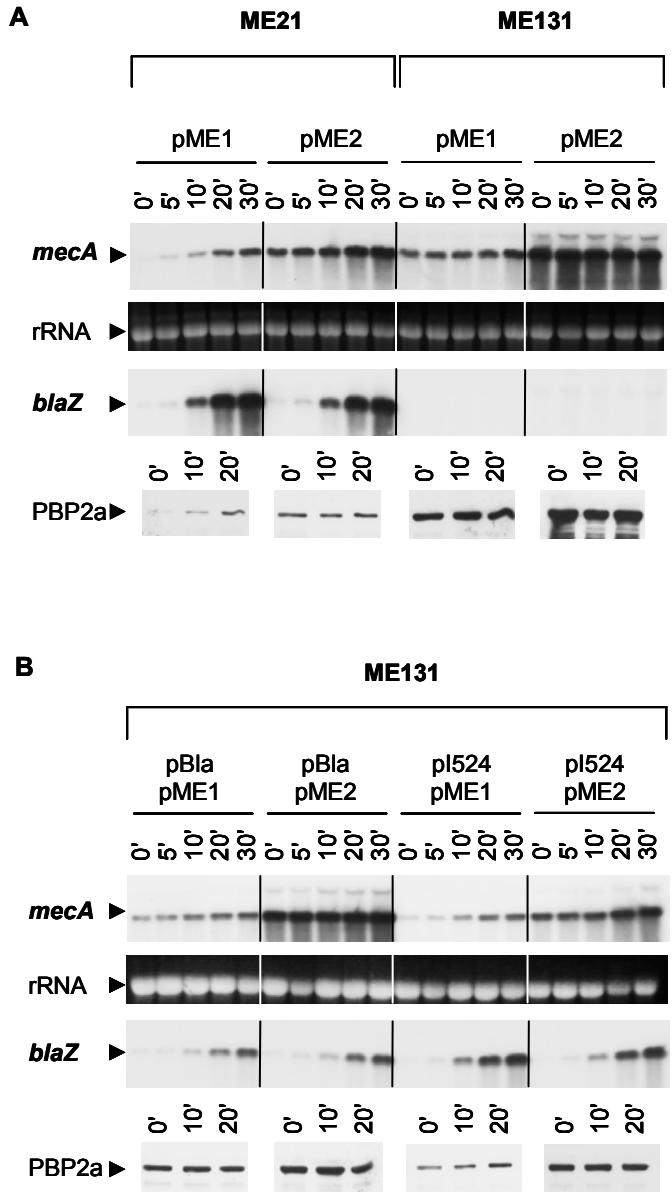

Figure 3.

Time course of *mecA*, *blaZ* and PBP2a production in different genetic backgrounds upon induction with a subinhibitory concentration of cefoxitin. A, strains ME21 or ME131 containing either pME1 or pME2; B, strain ME131 containing penicillinase plasmid pBla or pI524, and either plasmid pME1 or pME2. Sampling times after cefoxitin induction are indicated. Top, Northern blots probed with *mecA* and *blaZ*; bottom, Western blot of PBP2a. The quantitative loading of RNA is shown by 23 S rRNA.

Effect of *mecA* promoter mutation on resistance levels in different genetic backgrounds. The overall β-lactam resistance levels of all ME21 and ME131 transformants were essentially in the range of those of their respective wildtype parent strains before curing, irrespective of which plasmid they contained. Plasmids in the ME21 background yielded much lower values than in ME131 (Table 2). By Etest there were 2-4-fold differences in ampicillin MICs, with strains carrying pME1 being slightly less resistant than their counterparts carrying pME2 (Table 2). No relevant differences in oxacillin and cefoxitin MICs between isogenic transformants carrying pME1 and pME2 were observed in strain ME21; whereas in the COLn background of strain M131 all values were above 256 µg ml^{-1} by Etest, and small differences could only be seen qualitatively on gradient plates when extending the cefoxitin concentration over 256 µg ml^{-1} (Figure 4).

Table 2. Antibiotic minimal inhibitory concentration [µg ml^{-1}] and minimal doubling time [min].

strain	AM	FX	OX	MD
CHE482	2	8	1.5	25
ME21	0.75	4	0.38	25
ME21 pBUS1	1.5	4	0.38	32
ME21 pME1	3	12	0.75	32
ME21 pME2	6	16	0.75	31
ME21 pME3	8	16	0.75	28
COLn	24	>256	>256	46
ME131	0.19	4	0.75	45
ME131 pBUS1	0.19	4	0.75	46
ME131 pME1	4	>256	>256	51
ME131 pME2	24	>256	>256	54
ME131 pME3	24	>256	>256	47
ME131 pBla	1.0	4	1.0	44
ME131 pBla pME1	12	>256	>256	58
ME131 pBla pME2	48	>256	>256	63
ME131 pBla pME3	64	>256	>256	48
ME131 pI524	1.5	4	1.5	42
ME131 pI524 pME1	12	>256	>256	59
ME131 pI524 pME2	48	>256	>256	58
ME131 pI524 pME3	48	>256	>256	48

Abbreviations: AM, ampicillin; FX, cefoxitin; MD, minimal doubling time; OX, oxacillin.

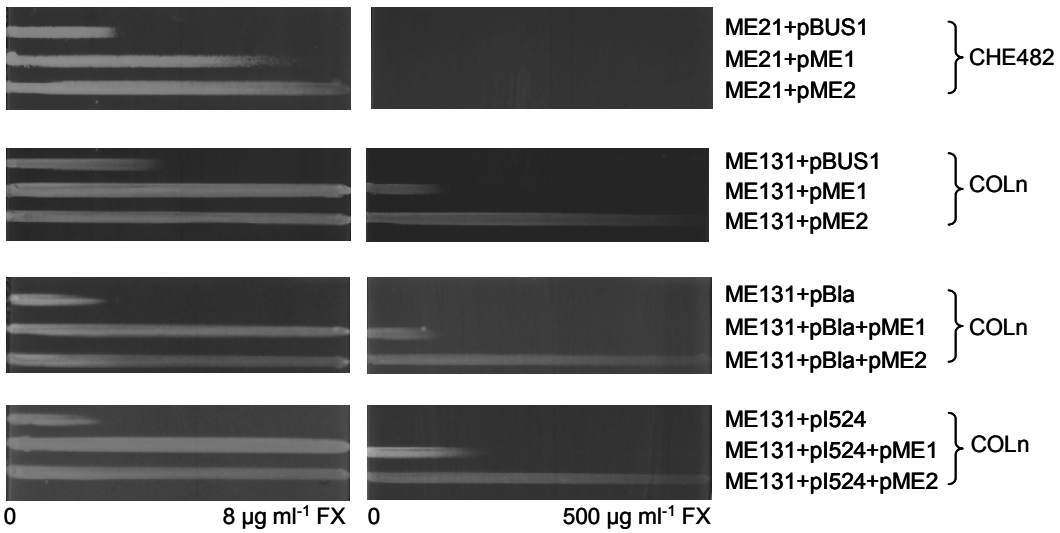

Figure 4.

Cefoxitin gradient plates. Strains were swabbed across agar plates containing an increasing concentration gradient of cefoxitin. Gradients of cefoxitin used were 0-8 µg ml^{-1} and 0-500 µg ml^{-1}.

Role of the β-lactamase operon on *mecA* expression and β-lactam resistance levels. Although the *mecA* promoter mutation in CHE482 had a considerable effect on *mecA* transcription, it only had a small effect on β-lactam resistance levels, indicating that the genetic backgrounds of CHE482 and COLn seemed to be the major factor controlling resistance levels. A key difference between these two genetic backgrounds was the presence of a β-lactamase plasmid in CHE482, which may restrict this strain to expressing low levels of resistance.

Attempts to cure the approximately 32-kb β-lactamase plasmid (pBla) from CHE482 were unsuccessful (data not shown). Therefore, pBla and a previously characterised β-lactamase plasmid pI524 (Murphy and Novick, 1980) were transformed into the ME131 isolates containing pME1 and pME2 (Figure 2). Northern blots confirmed that all resulting transformants contained an inducible *blaZ* gene, indicating that the BlaR1/BlaI regulatory elements were functional (Figure 3B). Introduction of the β-lactamase plasmids into ME131 had a clearly visible effect on *mecA* transcription, especially in pME1. Transcription of *mecA* from pME1 and pME2 adopted similar patterns to those seen in ME21. In the COLn background plasmid pI524 appeared to exert a stronger regulatory effect than pBla, resulting in stronger *mecA* repression before induction (Figure 3B).

Once again, Western blots were performed to show the correlation between *mecA* transcription and PBP2a production (Figure 3B). Although induction of *mecA* transcription did not always result in a corresponding induction of PBP2a expression, PBP2a signals were strongest in the strains with the highest levels of *mecA* transcription.

Changes in *mecA* expression, resulting from introduction of pBla and pI524, however, had no visible effect on cefoxitin resistance levels on gradient plates (Figure 4). Oxacillin and cefoxitin Etest MICs from all ME131 transformants remained very high, at >256 μg ml^{-1} (Table 2).

***mecA* ribosomal binding site mutation.** The recently sequenced SCC*mec* from another Zurich-isolated MRSA, strain ZH47 (Heusser et al., 2007), contained a different nucleotide substitution. This substitution of G to T in the ribosomal binding site (rbs) of *mecA*, 7-bp upstream of the ATG, occurs more frequently in database sequences. It is also found in the epidemic community MRSA clone USA300 and in several other SCC*mec* type IV strains. To determine if this alteration in the rbs influenced *mecA* expression, PBP2a production or β-lactam resistance levels, the *mecA* gene and promoter from ZH47 was cloned to produce plasmid pME3, and transformed into ME21, ME131, ME131 pBla and ME131 pI524 (Figure 2).

Oxacillin and cefoxitin MICs of strains containing pME3 were identical to those containing pME2 (Table 2). Ampicillin MICs were also generally the same except for in ME21 pME3 and ME131 pBla pME3, where they were less than 2-fold higher than their corresponding pME2-containing counterparts. Small differences in the resistance levels conferred by the three plasmids in ME21 could be seen by gradient plate, with pME1 providing the lowest and pME3 the highest level of resistance (Figure 5A).

In ME21, transcription of *mecA* from pME3 was induced by cefoxitin (Figure 5B) as was PBP2a expression. In direct transcript-level comparisons between ME21 strains containing pME1, pME2 and pME3, the highest levels of transcription were from pME2, then pME3 and the lowest from pME1 (data not shown). In ME131, ME131 pBla, and ME131 pI524, the transcription profiles of *mecA* from pME3 were virtually identical to those from pME2 (data not shown). Western blot analysis revealed a close correlation between *mecA* transcription levels and PBP2a production (Figure 5B).

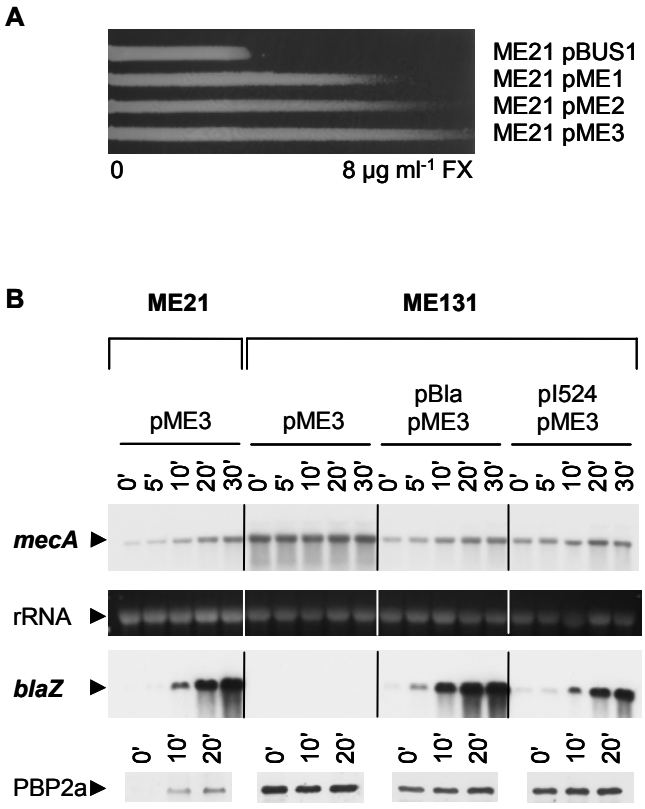

Figure 5.
A, Gradient plate of 0-8 µg ml^{-1} cefoxitin comparing the three *mecA* plasmids in the ME21 background. B, Northern blots of *mecA* and *blaZ* expression with the corresponding Western blots of PBP2a of plasmid pME3 in different genetic backgrounds in presence and absence of penicillinase plasmids as indicated. The time points of harvesting before and after induction with cefoxitin are indicated. The quantitative loading of RNA is shown by 23 S rRNA.

Growth rate differences. Growth curves were performed to determine the minimum doubling times for all strains (Table 2). The minimum doubling time for strain COLn was significantly higher (46 min) than that of CHE482 (25 min). Minimal doubling times were not significantly affected by curing of SCC*mec*, but were increased for both strains upon introduction of plasmids; in ME21 by introduction of both the empty plasmid and *mecA*-encoding plasmids and in ME131 only by introduction of the *mecA*-encoding plasmids. Plasmid pME3 appeared to impose the lowest burden in terms of growth rate, while pME1 and pME2 both imposed a similar, slightly higher burden. Subsequent introduction of plasmids pBla and pI524 into ME131 strains appeared to result in a small further increase in doubling time.

Discussion

Genetic factors governing β-lactam resistance levels are still poorly understood. Although it has been shown that there is no direct correlation between levels of *mecA* expression and levels of methicillin resistance, it has been shown that variable repressor activity, due to mutations in *mecI* and the *mecI* promoter can influence resistance levels (Kuwahara-Arai et al., 1996; Rosato et al., 2003a). Analysis of the mec complex from CHE482 revealed that it contained a novel *mecA* promoter mutation, which creates a perfect palindromic sequence covering the region containing both the -10 box of the *mecA* promoter and the MecI/BlaI repressor-binding operator region. Due to the location of the mutation it seemed likely that it could affect *mecA* transcription efficiency and/or repressor-binding, and hence be a contributing factor to the extremely low-level β-lactam resistance of CHE482. A database search revealed that this mutation was very rare, but was also present in strain WIS, and various SCC*mec* type V isolates from Australia, the majority of which also have low level oxacillin resistance levels (Ito et al., 2004; O'Brien et al., 2005). We also looked at the effect of a mutation in the *mecA* rbs, which has been previously described and associated with low-level resistance. Shukla et al., 2004, identified this rbs mutation in 34.7% of strains investigated during an epidemiological screen. All strains containing this mutation had a type IV SCC*mec* and the median oxacillin MIC of these strains was lower than in strains without the mutation (Shukla et al., 2004). However, the influence of this mutation on resistance levels has never been investigated experimentally.

A previous study in *S. sciuri* indicated the potential impact that promoter mutations could have on β-lactam resistance levels. *S. sciuri* contains a non-SCC*mec* associated *mecA* homologue, *mecA1*, which has an 88% similarity to the *mecA* gene from SCC*mec* in *S. aureus*. The *mecA1* gene did not confer β-lactam resistance in wildtype *S. sciuri* strain K1, however, a highly resistant variant, K1M200, could be selected in vitro. Increased resistance was accompanied by a mutation in the *mecA1* promoter. Even though *mecA1* does not contain a *mecA* consensus promoter, it was interesting to note that the *mecA1* -10 box in K1 (TATA**T**T) was identical to that in CHE482. In the highly resistant variant K1M200 the -10 box mutation to TATA**A**T was accompanied by an increase in oxacillin MIC from 1 to 200 µg ml^{-1}. Promoter-reporter gene fusions showed that the K1M200 promoter had a much higher activity than the K1 promoter (Wu et al., 2001). This suggests that alterations in the -10 box can have large impacts on transcription efficiency.

We established a system to test the influence of *mecA* promoter variants in two different *S. aureus* genetic backgrounds. The *mecA* genes and their respective promoters from CHE482 (-10 box mutation), ZH47 (rbs mutation) and COLn (consensus promoter) were cloned and introduced into CHE482 and COLn strains that had been cured of their SCC*mec* (Figure 2).

Resulting strains were then tested for *mecA* transcription and induction by cefoxitin, their corresponding PBP2a expression levels and their β-lactam resistance levels.

Transcriptional analyses revealed that the CHE482 promoter mutation, in pME1, had a strong effect on *mecA* transcription. In the CHE482 background (ME21), which contains the β-lactamase regulatory loci *blaR1/blaI*, transcription of *mecA* from pME1 was completely repressed and induced over time after addition of a sub-inhibitory concentration of cefoxitin. However, transcription of *mecA* from the consensus-sequence promoter in pME2 was already strong in the absence of induction and only increased slightly upon addition of cefoxitin. This indicates that the pME1 promoter mutation creates a much stronger BlaI repressor binding site than the general *mecA* promoter consensus. Analysis of the two promoter sequences in the COLn (ME131) background, which contains neither MecR1/MecI nor BlaR1/BlaI regulatory proteins, revealed further transcriptional differences. As expected, transcription of *mecA* was constitutive from both promoters, however, the transcript abundance from pME2 was much greater than that from pME1, indicating that transcription may be more efficient from the pME2 promoter. Transcription levels of *mecA* from pME3 were very similar to those from pME2, indicating that the *mecA* rbs mutation had no significant effect on transcription and functioned with a similar efficiency to the consensus-sequence promoter. PBP2a levels, from Western blot analyses, revealed that PBP2a expression levels closely corresponded to *mecA* transcription profiles, indicating that the ribosomal binding site mutation in pME3 appeared to have no effect on PBP2a translation.

The large effect that the pME1 promoter had on *mecA* transcription did not, however, result in a correspondingly large influence on β-lactam resistance levels. Visible differences in resistance could be seen on cefoxitin gradient plates, indicating that the different promoter sequences did have small effects on resistance. However, overall effects on MIC levels were negligible. All ME21 complemented strains had similar, low oxacillin and cefoxitin MICs to CHE482; and all ME131 complemented strains had oxacillin and cefoxitin MICs of >256 μg ml^{-1}. Therefore resistance levels in these two strains appear to be mainly governed by factors in their genetic background. Strains within identical clonal complexes have previously been shown to generally have similar resistance profiles (Katayama et al., 2005). CHE482 belongs to multi locus sequence type (MLST) ST45 and clonal complex 45, which is one of the so called major MRSA strain lineages (Katayama et al., 2005; Wannet et al., 2004). The majority of published strains belonging to this clonal complex, regardless of the SCC*mec* type they carry, including types I, II, IV, V or a novel SCC*mec* type (Boyle-Vavra et al., 2005; Enright et al., 2002; Ip et al., 2005; O'Brien et al., 2005; Wannet et al., 2004), are low-level resistant (≤ 32 μg ml^{-1}) with only a few exceptions (Regev-Yochay et al., 2006; Wannet et al., 2004). By comparison strain COL, belonging to MLST ST250 and containing a type I

SCC*mec* (Enright et al., 2002; McAleese et al., 2005) is associated with higher resistance levels than ST45.

Because resistance levels of the plasmid-complemented strains were very similar to the wildtype strains before curing, it can be assumed that no other genes encoded on the SCC*mec* elements influence resistance levels, as previously suggested by Ryffel et al. (Ryffel et al., 1994). These results also confirmed previous findings that *mecA* transcription levels do not directly correlate with methicillin resistance levels (de Lencastre et al., 1994).

A key difference between the genetic backgrounds of CHE482 and COLn is the presence of the BlaR1/BlaI regulators in CHE482. Unfortunately, attempts to cure CHE482 of its β-lactamase-encoding plasmid were unsuccessful, so we were unable to determine what role BlaI repression played in the low level resistance of this strain. We therefore transformed the β-lactamase plasmid (pBla) from CHE482 and another β-lactamase plasmid pI524 into ME131. The presence of β-lactamase plasmids, especially pI524, in most cases led to the repression of *mecA* and subsequent induction upon cefoxitin exposure. The unequal repression by pI524 and pBla is possibly caused by differences in the *blaI* sequences (data not shown, (Smith and Murray, 1992)), which differ in two amino acids, that could potentially have an influence on dimerisation efficiency/ bonding force and therefore binding intensity of BlaI to the *mecA* promoter sequence. Other factors which might be involved are the plasmid copy number and the transcription efficiency. These changes in *mecA* transcription induced by pI524/pBla, however, once again had little effect on altering β-lactam resistance levels.

Another major difference between CHE482 and COLn is their growth rates. CHE482 is a very fast growing strain with a minimum doubling time of 25 min. It had been postulated that *mecA* is repressed in most strains by the MecR1/MecI or BlaR1/BlaI, because high levels of PBP2a production in the absence of β-lactams is unfavourable (Rosato et al., 2003b). Therefore it is likely that strains such as COLn, which constitutively produce PBP2a, have undergone genetic mutations or adaptations allowing it to tolerate consistently high levels of PBP2a. The price for these adaptations might be reflected in its relatively slow growth rate. The hypothesized link between methicillin resistance levels and fitness (Ender et al., 2004) was also supported by the decreased growth rate of in vitro-selected CHE482 isolates with high oxacillin resistance levels (data not shown). The promoter mutation in CHE482, leading to tightly repressed PBP2a in the absence of β-lactams, may have been selected as a fitness adaptation of this strain.

In summary, it appears that although *mecA* promoter mutations can have a large influence on *mecA* transcription and corresponding PBP2a levels, they have little effect on β-lactam resistance levels. Resistance levels appear to be strictly governed by unknown factors within a strains genetic background.

Acknowledgement

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3.3 Project 3: A novel DNA-binding protein modulating β-lactam resistance in Staphylococcus aureus

Abstract

Methicillin resistance in *Staphylococcus aureus* is conferred by the *mecA*-encoded penicillin-binding protein PBP2a, which is regulated by the repressor/sensor transducer MecI/MecR1 and/or BlaI/BlaR1. Several additional chromosomal factors are also known to influence the expression of methicillin resistance, including some hypothetical factors that are yet to be identified. A previously uncharacterized protein, SA1665, of unknown function, containing a helix-turn-helix motif characteristic of DNA-binding transcriptional regulators, was found to bind to the *mecA*-promoter/5'-*mecA* coding sequence. Nonpolar deletion of SA1665 in several methicillin-resistant *S. aureus* (MRSA) of different genetic backgrounds increased oxacillin resistance to varying extents in all but one of the mutants; the remaining mutant derived from a highly β-lactam resistant strain had decreased resistance. Deletion of SA1665, however, had no obvious effects on *mecA* transcription or its induction by β-lactams, or on amounts of PBP2a produced, in any of the strains tested. Therefore, despite the proven binding of SA1665 to the *mecA*-promoter/5' region of the *mecA* gene, evidence suggests that its influence on β-lactam resistance expression is not exerted via transcriptional control of *mecA*, but through the control of currently unknown chromosomal factors.

Introduction

Methicillin resistant *S. aureus* (MRSA) are an ever increasing threat in clinical settings and more recently as an emerging community acquired pathogen. Their invasiveness and pathogenesis depends on a variable arsenal of virulence factors, while their resistance to β-lactams and ability to rapidly generate or take up additional resistance determinants severely hampers therapy and eradication. In *S. aureus*, β-lactam resistance can be conferred by two mechanisms, either by production of a penicillinase, which hydrolytically cleaves β-lactam antibiotics, or by production of an additional penicillin-insensitive penicillin-binding protein (PBP), PBP2a (19, 23, 34). PBP2a takes over the transpeptidase function of native PBPs, which are acylated in the presence of β-lactam antibiotics rendering them inactive. β-lactam antibiotics act as substrate analogs, binding to the active site Ser403 at the N-terminal transpeptidase domain of the PBPs (39). PBP2a has a decreased affinity for β-lactams due to a distorted active site, which has to undergo novel conformational changes reducing the acylation rate and allowing peptidoglycan crosslinking to continue (25). In the presence of β-lactams, PBP2a maintains cell wall biosynthesis together with PBP2, a bifunctional enzyme containing a β-lactam-sensitive transpeptidase domain, and a β-lactam-insensitive trans-glycosylase domain responsible for polymerization of the peptidoglycan disaccharide subunits (32).

PBP2a is encoded by *mecA*, a gene divergently transcribed from its cognate regulators, *mecR1* (signal/sensor transducer) and *mecI* (transcriptional repressor). If *mecR1/mecI* are absent or truncated, transcriptional control of *mecA* is taken over by the structurally similar *blaZ* (penicillinase) regulatory elements *blaR1/blaI*, if present. In the absence of both regulatory loci, *mecA* is constitutively transcribed. BlaI and MecI both bind as homodimers to two sites within the *mecA* promoter/operator region, inhibiting transcription of both *mecA* and *mecR1/mecI* (17, 38). In the presence of β-lactams, the transmembrane proteins BlaR1/MecR1, undergo a conformational change at the C-terminal penicillin-binding domain due to acylation of the active site serine. This is followed by autoproteolytic cleavage of the N-terminal cytoplasmic domain, leading to the activation of the cytoplasmic peptidase and subsequent dissociation of the repressor due to proteolytic degradation of the repressor dimer (13, 16, 42). However, the signal transduction cascade of this regulatory system has still not been completely elucidated. Cohen and Sweeney postulated that an unidentified genomic factor, *blaR2*, was essential for the regulation of *blaZ*, as its mutation resulted in constitutive *blaZ* production (8). A number of additional genomic factors essential for methicillin resistance (*fem*), including genes involved in peptidoglycan precursor formation and hydrolysis, teichoic acid synthesis, global regulators and genes of unknown or poorly characterised functions, have been shown to influence methicillin resistance levels (3, 9, 10, 36).

To identify potential factors involved in *mecA* regulation we performed DNA-binding protein purification assays using the *mecA* promoter region/5' coding region of *mecA* as the bait. A putative DNA-binding protein, SA1665, was shown to bind to this DNA fragment and subsequently shown to have strain-dependent effects on β-lactam resistance levels when deleted.

Materials and Methods

Strains mutants. Strains and plasmids used in this study are listed in Table 1. Clinical isolates are from the IMM collection in Zurich, Switzerland. Strains were grown at 37°C in Luria Bertani (LB) broth or on LB agar. Media were supplemented with the following antibiotics when appropriate: 25 or 50 µg ml^{-1} kanamycin, 10 µg ml^{-1} chloramphenicol, 5 or 10 µg ml^{-1} tetracycline, 100 µg ml^{-1} ampicillin, 1-2048 µg ml^{-1} oxacillin.

Table1. Strains and plasmids used in this study.

Strain	Relevant genotype	Origin, Reference
S. aureus		
CHE482	clinical MRSA isolate, CC45, ST45, SCC*mec*$_{N1}$, *blaZ* (pBla)	(11, 33)
ΔCHE482	CHE482 ΔSA1665	this study
COLn	COL derivative cured of plasmid pT181 (*tetK*), SCC*mec* type I	(22)
ΔCOLn	COLn ΔSA1665	this study
ZH37	clinical MRSA isolate, CC45, ST45, SCC*mec* type IV, *blaZ*	(33)
ΔZH37	ZH37 ΔSA1665	this study
ZH44	clinical MRSA isolate, SCC*mec* type II, *aac-aph*	(33)
ΔZH44	ZH44 ΔSA1665	this study
ZH73	clinical MRSA isolate, SCC*mec* type IV, *blaZ*	(33)
ΔZH73	ZH73 ΔSA1665	this study
RN4220	NCTC8325-4, restriction negative	(24)
E. coli		
DH5α	restriction-negative strain for cloning	Invitrogen
BL21 (DE3)	F^- *omp*T *hsd*S_B($r_B^-m_B^-$) *gal dcm* (DE3)	Novagen
Plasmids		
pBUS1	*S. aureus-E. coli* shuttle vector, *tetL*	(37)
pAW17	*S. aureus-E. coli* shuttle vector, *aac-aph*	(37)
pKOR1	*S. aureus-E. coli* shuttle vector, *cat, bla*	(2)
pME17	pKOR1-SA1664/SA1666, *cat*	this study
pET28nHis$_6$	*E. coli* protein expression vector, with n-terminal His$_6$ tag, *aac-aph*	unpublished D. Frey
pME20	pET28nHis$_6$-SA1665, *aac-aph*	this study
pME26	pAW17-SA1665 and 700 bp up- and 380 down-stream, *aac-aph*	this study
pME27	pBUS1-SA1665 and 700 bp up- and 380 bp down-stream, *tetL*	this study

Abbreviations: CC, clonal complex; ST, sequence type.

Susceptibility testing. Minimal inhibitory concentrations (MIC) were determined by Etest (Solna) according to CLSI guidelines (7). Resistance comparisons were performed using gradient plates, whereby cell suspensions of 0.5 McFarland were swabbed across agar plates containing an increasing concentration gradient of oxacillin. To ensure plasmid

maintenance kanamycin (25 µg ml^{-1}) or tetracycline (5 µg ml^{-1}) was added where appropriate. Population analysis profiles of oxacillin resistance were determined from overnight cultures grown in LB. Dilutions ranging from 10^0 to 10^8 were plated on agar plates of increasing oxacillin concentrations (0-2048 µg ml^{-1}) and incubated at 35°C for 48 h.

Markerless deletion of SA1665. In frame markerless deletions of SA1665, from the chromosomes of CHE482, ZH37, ZH44, ZH73 and COLn were constructed using the pKOR1 plasmid system as described by Bae et al. (2). Primer pairs used to amplify the DNA fragments flanking SA1665, for recombination into pKOR1 were: me62attB1/me51BamHI and me62BamHI/me62attB2 (Table 2). Deletion mutants were confirmed by Southern blot analysis (1) and pulsed field gel electrophoresis (PFGE) (41).

Cloning of SA1665 for complementation. A 1533-bp DNA fragment, containing SA1665 together with 690-bp of upstream and 379-bp of downstream DNA, was amplified using primers me94BamHI/me94Asp718 (Table 2) and cloned into the *E. coli/S. aureus* shuttle vectors pAW17 and pBUS1, creating the complementing plasmids pME26 and pME27, respectively. Plasmids were then electroporated into RN4220 and further transduced using phage 85α.

Binding-protein purification. Crude protein extracts were isolated from 50 ml cultures, grown to OD_{600nm} 1.5, using Lysing Matrix B (BIO 101 Systems). Cell pellets were resuspended in 1.5 ml PBS (pH 7.4) and shaken 3x 20 s at speed setting 6.0 in a FastPrep FP120 (BIO 101 Systems). Suspensions were centrifuged for 1 min at 12,000 x *g*, the supernatants were collected and centrifuged for 10 min at 20,000 x *g* at 4°C. Supernatants were transferred to Amicon Ultra Centrifugal Filter Devices (Millipore), washed with 1x binding buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM DTT, 0.5 M NaCl) and centrifuged until the volume had reduced to ~120 µl. Protein concentrations were measured by Bradford assay (BioRad Laboratories GmbH) (5). For binding protein purification, a biotinylated *mecA* promoter DNA fragment was amplified using primers me36F/me36Rbiot (Table 2) and 10 pmol was bound to streptavidin coated magnetic beads (10 mg ml^{-1}, Dynabeads M-280 Streptavidin, DYNAL BIOTECH) that had been pre-washed twice with 2x BW buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 2 M NaCl) and resuspended in 400 µl of 1x BW buffer. After rotating at room temperature for 30 min, the beads were washed once with 1x BW buffer and twice with 1x protein binding buffer (20 mM Hepes, pH 7.6, 1 mM EDTA, 10 mM $(NH_4)_2SO_4$, 1mM DTT, 0.2% Tween20 (w/v), 30 mM KCl). DNA-coated beads were mixed with 100 µg of crude protein extract in the presence of 1x protein binding buffer, 0.02 µg $µl^{-1}$ poly d(I-C) and 2 ng $µl^{-1}$ poly L-lysine, and incubated at room temperature for 30 min

with constant rotation. After binding, beads were washed 4 times in 1x protein binding buffer and bound proteins were eluted in 50 µl elution buffer (1x protein binding buffer containing 2 M KCl). Eluted proteins were dialysed over night at 4°C against water in ElutaTubes (Fermentas). Samples were concentrated by evaporation, run on a 15% SDS polyacrylamide gel and silver stained using the Protein Silver Staining kit (Amersham Biosciences AB) without the addition of glutaraldehyde.
Bands of interest were excised from gels and analysed by mass spectrometry (LC/ESI/MS/MS) by the Functional Genomics Centre Zurich. The SA1665 protein sequence [BAB42933] was analysed by Blast search (http://www.ncbi.nlm.nih.gov/ BLAST) and a motif search (http://myhits.isb-sib.ch/cgi-bin/motif_scan).

Primer extension. RNA was extracted (6) from CHE482 cultures that were grown to OD_{600nm} 0.5. Primer extension reactions were performed using 20 µg of total RNA and 3 pmol of the 5'-biotin-labeled primer me97 (Table 2) using Superscript II reverse transcriptase (Invitrogen), according to the manufacturer's instructions. Primer me97 was also used to generate sequencing reactions using the Thermo Sequenase sequencing kit (U.S. Biochemicals) and a sequencing template amplified using primers me52F and me52R (Table 2). All samples were then run on a 6 % polyacrylamide sequencing gel, contact blotted onto a positive charged nylon membrane and biotin-detection was performed using the Biotin Chromogenic Detection Kit (Fermentas).

Expression of recombinant SA1665 protein. SA1665 was amplified using primers me65BamHI/me65XhoI (Table 2) and cloned in-frame into pET28nHis_6 (unpublished, D. Frey). The resulting plasmid, pME20, was then transformed into *E. coli* BL21 and recombinant nHis_6-SA1665 protein was harvested. To maximise the abundance of soluble protein produced, cells were grown in osmotic shock medium (1 g l^{-1} NaCl, 16 g l^{-1} tryptone, 10 g l^{-1} yeast, 1 M sorbitol, 10 mM betaine, modified from (4)) to OD_{600nm} 0.5 at 37°C then cooled briefly on ice. Recombinant protein expression was induced by addition of 100 µM IPTG and the cultures were grown overnight at 22°C. Crude soluble proteins were extracted from cell pellets using CelLyticB 2X Cell Lysis Reagent (SIGMA). HIS-Select Cobalt Affinity Gel (SIGMA) was used to purify recombinant nHis_6-SA1665 according to the manufacturer's instructions.

Electro mobility shift assay. For gel shift assays, 6 ng aliquots of the biotinylated-DNA fragment used for binding-protein purification were incubated with 0-250 ng of purified nHis_6-SA1665 protein in 1x binding buffer (20 mM Hepes pH 7.6, 1 mM EDTA, 10 mM $(NH_4)_2SO_4$, 1mM DTT, 0.2 % Tween20 (w/v), 30 mM KCl) containing 0.05 µg $µl^{-1}$ poly d(I-C) (Roche) and

5 ng μl^{-1} poly L-lysine (Roche)). For control binding reactions, 130 x unlabelled *mecA* promoter DNA (amplified using primers 36F/36R, Table 2) was used as a specific binding competitor and 6 ng of herring sperm DNA was used as unspecific competitor DNA. Binding was carried out at 22°C for 30 min and samples were run on 6 % native polyacrylamide gels at 70 V. Samples were contact blotted onto positive charged nylon membrane, UV cross-linked and detected with the Biotin Chromogenic Detection Kit (Fermentas).

DNase I footprinting. A 193-bp biotinylated DNA fragment of the *mecA* promoter was amplified with primers me96 and me36F (Table 2). DNA-protein binding reactions, using 40 ng of biotinylated PCR product and 1200-1500 ng of purified $nHis_6$-SA1665 protein, were performed using the LightShift Chemiluminescent EMSA kit (Pierce Biotechnology) according to the manufacturer's instructions. Binding reactions were incubated for 30 min at 22°C, before being subjected to DNAse I digestion (0.05 U μl^{-1}, Fermentas) for 1 min at RT. An equal volume of stop solution (0.6 M NaOAc, 25 mM EDTA, MS2 phage RNA 40 $\mu g\ ml^{-1}$, pH 4.8) was then added to terminate DNase I digestion (18, 21). Samples were then ethanol precipitated, resuspended in an appropriate volume of dH_20 and separated on a 6 % sequencing gel. To identify potentially protected DNA regions, samples were run alongside a negative control consisting of 40 ng of biotinylated-DNA subjected to identical processing but in the absence of protein. Gels were contact blotted onto positively charged nylon membrane (SIGMA) and biotin-detection was performed using the LightShift Chemiluminescent EMSA kit (Pierce Biotechnology). The associated sequencing reactions were carried out using the biotinylated primer me96 and the Thermo Sequenase sequencing kit (U.S. Biochemicals).

Northern blot analysis. Strains were grown overnight in LB (Difco), diluted 1:200 and grown for another 3 h. This preculture was then used to inoculate 150 ml (1:1,000) of fresh prewarmed LB. Cells were then grown at 37°C to OD_{600nm} 1.0 and induced with cefoxitin 4 $\mu g\ ml^{-1}$. Cultures were sampled at time point 0' before induction and at 10' and 30' after induction. To monitor SA1665 expression over growth, separate cultures were also sampled at different growth stages corresponding to OD_{600nm} 0.25, 0.5, 1, 2, and 4. Samples were harvested by centrifugation at 4°C for 3 min at 13,000 g then snap-frozen in liquid nitrogen. Total RNA was extracted as described in Cheung et al. (6). RNA samples (8 µg) were separated in a 1.5 % agarose-20 mM guanidine thiocyanate gel in 1x TBE running buffer (15), transferred and detected as described previously (27). Digoxigenin (DIG) labelled-probes were amplified using the PCR DIG Probe synthesis kit (Roche) and the following primers: for *mecA*, mecAP4/mecAP7 (30); for *blaZ*, blaZF/blaZR; for SA1665, SA1665F/SA1665R; for *mecR1'*, mecR1F/ mecR1R; for SA1664, SA1664F/ SA1664R; for SA1666, SA1666F/ SA1666R and for SA1667, SA1667F/ SA1667R (Table 2).

Western blot analysis. Cells were cultured, as previously described for Northern blot analysis, to OD_{600nm} 1.0 then induced with cefoxitin 4 µg ml^{-1}. Samples were collected at 0' (before induction), 10' and 30' (after induction), harvested by centrifugation at 13,000 g for 3 min, resuspended in 50 µl of PBS pH 7.4 containing 150 µg ml^{-1} of DNase, lysostaphin and lysozyme and incubated for 1 h at 37°C. Suspensions were then sonicated in a sonicating-water bath for 4x 10 sec. Protein aliquots (15 µg) were separated on 7.5 % SDS-polyacrylamide gels, blotted onto nitrocellulose membrane (Hybond) and stained with Ponceau to confirm equal protein loading. For detection, membranes were blocked for 1 h in 1x low salt buffer (77.5 mM NaCl, 4.5 mM Tris, 0.05 % Tween20, pH 7.4) containing 5 % milk powder and then in 0.5 % milk powder in low salt buffer containing 40 µg ml^{-1} human IgG (CALBIOCHEM) for 30 min, before PBP2a antibody (1:20,000, Denka Seiken) was added and membranes were incubated for 1 h. Membranes were washed 3x 5 min in 1x low salt buffer before the secondary antibody, an anti-mouse IgG, HRP conjugate (1:2,500) in 1x low salt buffer containing 0.5 % milk powder, was added and incubated for 1 h. Membranes were washed 3x 5 min in 1x low salt buffer and detection was performed using SuperSignal West Pico Chemiluminescent Substrate (Pierce).

Table 2. Oligonucleotide primers used in this study.

Primer name	Nucleotide sequence (5'-3')	Reference
Markerless deletion		
me62attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCACTGGCTTATTCGCTTGA	This study
me51BamHI	ATTAGGATCCTTAGTACATATCTAGGCCTA	This study
me62BamHI	ATTAGGATCCACTCTGTCTATCCATTCTGT	This study
me62attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTTGTGCGACAAGGATTGCGA	This study
Cloning		
me94BamHI	ATTAGGATCCTCTTCAATCACTTGGCCAAT	This study
me94Asp718	ATTAGGTACCAAGGTGCTGATGGTTATGAA	This study
me65BamHI	ATTAGGATCCGATAGACAGAGTTTTACAGA	This study
me65XhoI	ATTACTCGAGGATATGTACTAATTCTTCTT	This study
Protein-DNA binding, EMSA and DNase I footprinting		
me36F	TGATAACACCTTCTACACCT	This study
me36Rbiot	BIOT-AACCCGACAACTACAACTAT	This study
me36R	AACCCGACAACTACAACTAT	This study
me96	BIOT-CTTTATCTTTGGAAGCATAA	This study
Primer extension		
me97	BIOT-ACTCTGTCTATCCATTCTGT	This study
me52F	CCACTGGCTTATTCGCTTGA	This study
me52R	TGTGCGACAAGGATTGCGAT	This study
Gene/transcript detection		
mecAP4	TCCAGATTACAACTTCACCAGG	(30)
mecAP7	CCACTTCATATCTTGTAACG	(30)
SA1665F	TTCGTATAGAGGCTGGTTAG	This study
SA1665R	AATTGGTTGGTTATCTGGAT	This study
mecR1F	TGACACGACTTCTTCGGTTA	This study
mecR1R	AACGTATATGTTCATGGCGA	This study
SA1664F	TCAGCATGTAGATAACGCAA	This study
SA1664R	ATGTCACAATTGTTCTTGCT	This study
SA1666F	GACCATTATATTGTGCGACA	This study
SA1666R	TTGTGCCTTAGGATGTATCA	This study
SA1667F	TTGTGCCTTAGGATGTATCA	This study
SA1667R	TAATACCGTGTGATGAAGCT	This study

Restriction sites are underlined; BIOT: end-labelled with biotin.

Results and Discussion

Identification of SA1665. A DNA-binding protein purification assay was performed to identify new potential factors involved in the regulation of *mecA*/PBP2a. The *mecA/mecR1* intergenic DNA region including the 5' 9 bp of *mecR1* and the first 52 bp of *mecA* was used as bait against crude protein extract from *Staphylococcus aureus* CHE482. Proteins binding to this DNA fragment were analysed by SDS-PAGE. Several protein bands appeared to be unspecific as they were pulled down by both DNA-labelled and unlabelled beads, however one protein band of approximately 15-20 kDa was only isolated from the labelled beads (Figure 1A).

This binding-protein was identified as a hypothetical protein of unknown function, encoded by the open reading frame (orf) SA1665 in the *S. aureus* N315 genome annotation [BA000018].

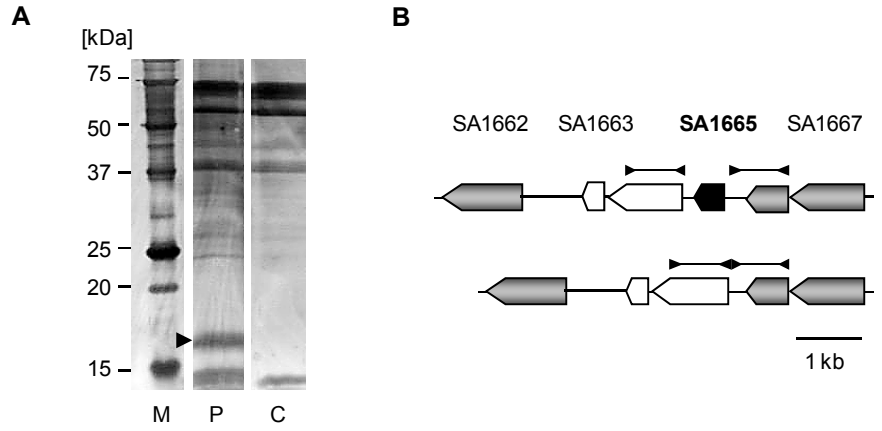

Figure 1.

A, Silver stained SDS-polyacrylamide protein gel of DNA-binding protein assay. One additional band, indicated by an arrow, was identified, in the positive sample (P) compared to the negative control (C). The protein size marker (M) is shown on the left as a reference.

B, Organisation of the putative operons covering SA1662 to SA1667. The regions used to construct the deletion mutants are indicated by lines framed by arrows, which represent the primers used for amplification. The chromosomal organisation after deletion of SA1665 is shown underneath.

SA1665 encodes a predicted 17-kDa protein with an N-terminal helix-turn-helix (HTH) motif characteristic for DNA-binding transcriptional regulators. According to the hypothesis of Pérez-Rueda et al., the position of the HTH motif in SA1665 indicates that it is most likely to act as a repressor (31). The amino acid sequence identity of SA1665 was 100 % conserved amongst *S. aureus* database sequences and 96-97 % conserved amongst other staphylococci, including *S. haemolyticus*, *S. epidermidis* and *S. saprophyticus*, indicating that the amino acid sequence of SA1665 is highly conserved amongst staphylococcal strains. There are no highly conserved homologs in other bacterial species; the most similar

homologs from *Bacillus licheniformis* DSM13 and *Desulfitobacterium hafniense* Y51 share only 64 % and 59 % amino acid sequence similarity, respectively, with SA1665.

Electro mobility shift assay (EMSA) using the *mecR1/mecA* intergenic region. EMSA was used to confirm SA1665 binding to the *mecA* promoter region. Crude protein extracts of *E. coli* strain BL21, carrying the empty plasmid (pET28nHis$_6$) and of BL21 containing pME20 (pET28nHis$_6$-SA1665) which expressed SA1665 upon induction with IPTG, were incubated with the 161-bp biotinylated DNA fragment previously used for protein-binding purification. A band shift was observed with extracts from the strain expressing recombinant nHis$_6$-SA1665 but not from the control strain carrying the empty plasmid. Several bands resulted from the shift, which is most likely due to protein oligomerisation (Figure 2A). The specificity of the gel shift was also demonstrated by the addition of increasing concentrations of purified nHis$_6$-SA1665 protein to the biotinylated DNA fragment (Figure 2B). Controls consisted of the biotinylated *mecA* promoter fragment with no protein, with 250 ng of protein in the presence of a 130 x excess of specific competitor DNA (unlabelled *mecA* promoter DNA) and with 250 ng of protein in the presence of unspecific competitor DNA (herring sperm DNA). Band-shift of the biotinylated DNA was inhibited in the presence of specific competitor DNA but not by the presence of the non-specific competitor DNA, confirming that nHis$_6$-SA1665 had a specific binding affinity for the 161-bp *mecA* promoter/5' *mecA* coding sequence fragment.

DNAse I protection assay. DNaseI footprinting, used to identify the portion of the *mecA/mecR1* intergenic region bound by nHis$_6$-SA1665 fusion protein, revealed a very small potentially protected region of only 3-bp (TAA) (Figure 2C). This region was consistently protected in three independent experiments. This region is much smaller than that of conventional DNA-binding transcription factor binding sites, but as this was the only region consistently protected from DNaseI digestion it potentially indicates the vicinity of nHis$_6$-SA1665 binding. Interestingly this protected region was not within the *mecA* promoter, but in the *mecA* coding sequence, 15-17 bp downstream of the translational start site of the *mecA* gene. PRODORIC (28) predicted a monomeric state for SA1665, which could explain why the potential binding site shown here contained neither a second protected region nor a palindromic sequence.

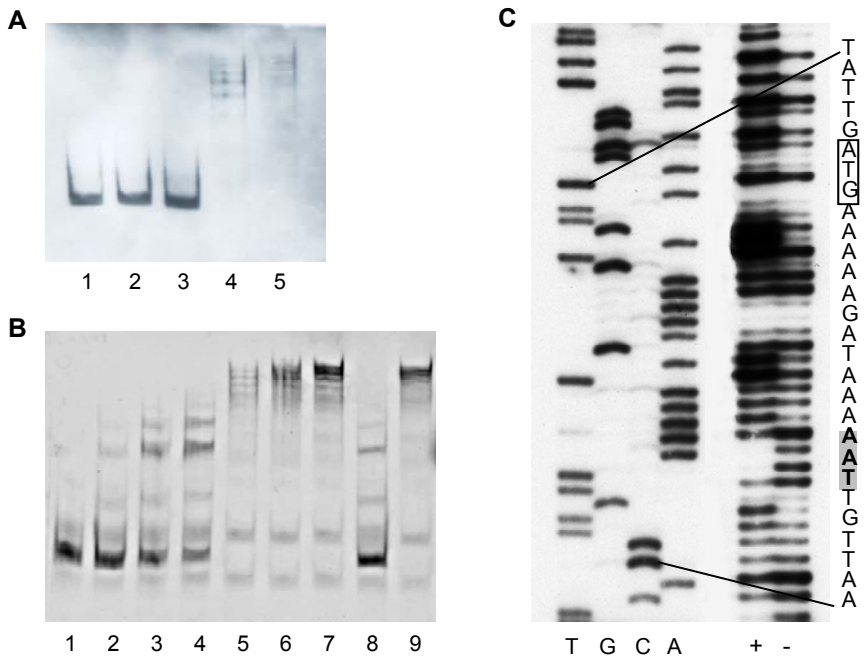

Figure 2.

Electromobility shift and DNase I footprinting of the *mecA-mecR1* intergenic region by SA1665.

A, Gel shift using crude protein extract. Lane 1, DNA, negative control; lanes 2 and 3, DNA with 200 ng and 500 ng of crude protein extract from *E. coli* BL21 pET28nHis$_{6,}$ respectively; lanes 4 and 5, DNA with 200 ng and 500 ng of crude protein extract from *E. coli* BL21 pME20 expressing SA1665, respectively.

B, Gel shift by purified SA1665 protein. Lane 1, negative control, DNA; lane 2, DNA with 10 ng protein; lane 3, DNA with 40 ng protein; lane 4, DNA with 75 ng protein; lane 5, DNA with 150 ng protein; lane 6, DNA with 200 ng protein; lane 7, DNA with 250 ng protein; lane 8, DNA with 250 ng of protein, in the presence of a 130 fold excess of unlabelled specific competitor DNA; lane 9, DNA and 250 ng protein in the presence of unspecific herring sperm DNA.

C, DNase I protection assay. A small region of 3-bp (highlighted in grey), just downstream of the *mecA* start codon (framed) was protected from DNase I digestion in the DNA sample incubated with nHis$_6$-SA1665 fusion protein (+) but not in the negative control which contained no protein (-).

Effect of SA1665 deletion on β-lactam resistance. The MRSA strains CHE482, which is the type strain of the Swiss drug clone, COLn, and three clinical MRSA strains (ZH37, ZH44 and ZH73) from the IMM collection of the University Zurich, were selected for the creation of SA1665 markerless deletion mutants via allelic replacement using pKOR1 (2) (Figure 1B). Strains CHE482, ZH37 and ZH73 all contained type B mec complexes (IS*1272*-Δ*mecR1-mecA*) with a truncated *mecI*/*mecR1* regulatory locus. In all three of these strains *mecA* transcription was regulated by the penicillinase sensor and repressor BlaI/BlaR1. ZH44 contained a type A mec complex (*mecI-mecR1-mecA*) whereby *mecA* expression was controlled by its cognate regulators MecI/MecR1. COLn contained neither the *mecR1/mecI* nor the *blaR1/blaI* regulatory loci, therefore *mecA* expression was constitutive. Further

relevant features of the selected strains were as follows: CHE482, belongs to clonal complex (CC) 45 and sequence type (ST) 45, carries a novel SCC*mec* (SCC*mec*$_{N1}$ (11)) and has a cefoxitin MIC of 12 µg ml^{-1}; COLn has a type I SCC*mec* and expresses high homogeneous oxacillin resistance and has a cefoxitin MIC of >256 µg ml^{-1}; ZH37 belongs to the same CC45/ST45 genomic background as CHE482 but contains a type IV SCC*mec* and has a cefoxitin MIC of 12 µg ml^{-1}; ZH73 (ST217, CC22) contains a type IV SCC*mec*, and has a cefoxitin MIC of 64 µg ml^{-1}; ZH44 has a similar PFGE profile to ST225, CC5 strains, has a type II SCC*mec* and a cefoxitin MIC of 48 µg ml^{-1}.

Resistance levels of SA1665 deletion mutants (ΔCHE482, ΔZH37, ΔZH44, ΔZH73 and ΔCOLn) and their corresponding wildtype parent strains were compared qualitatively on gradient plates containing appropriate increasing oxacillin concentration gradients (Figure 3A). All but one of the mutants had increased resistance levels compared to their parent, with ΔZH44 and ΔZH73 expressing considerably higher resistance levels. In contrast, the oxacillin resistance level of ΔCOLn was slightly lower than that of its parent. To quantify resistance differences between mutant and wildtype strains, population analysis profiles were performed. The resistance profiles of mutants ΔCHE482, ΔZH37, ΔZH44 and ΔZH73 all showed a distinct shift at the top of the curve, indicating that the majority of the cells in the mutant populations expressed higher basal oxacillin resistance levels than their corresponding wildtype strains (Figure 3B). Strains ΔCHE482 and ΔZH37 also had more highly resistant subpopulations than their respective parents. In contrast, ΔZH73 and its parent ZH73 both grew to the same oxacillin concentration limit and despite the majority of the ΔZH44 population having higher resistance than wildtype ZH44, the ZH44 parent strain actually segregated a more highly oxacillin resistant subpopulation than its mutant. This decrease in growth at maximal oxacillin concentrations appears to be a strain specific phenotype associated with SA1665 deletion. In the high homogeneously resistant strain COLn, SA1665 deletion also lowered resistance compared to the wildtype.

The identical resistance profiles of CHE482/ΔCHE482 and ZH37/ΔZH37 suggests that despite having different SCC*mec* elements, it is their common clonal background (CC45) that controls their resistance levels and the extent of resistance increase upon SA1665 deletion. Most of the currently known *fem/aux* factors reduce methicillin resistance levels when inactivated. Only a few genes, such as *lytH*, the *dlt* operon, *norG*, *sarV* and *cidA* increase resistance levels upon inactivation or mutation. All of these genes, except *norG* which is an efflux pump regulator, play a role in either autolysis or are important for cell physiology and growth (14, 26, 29, 35, 40).

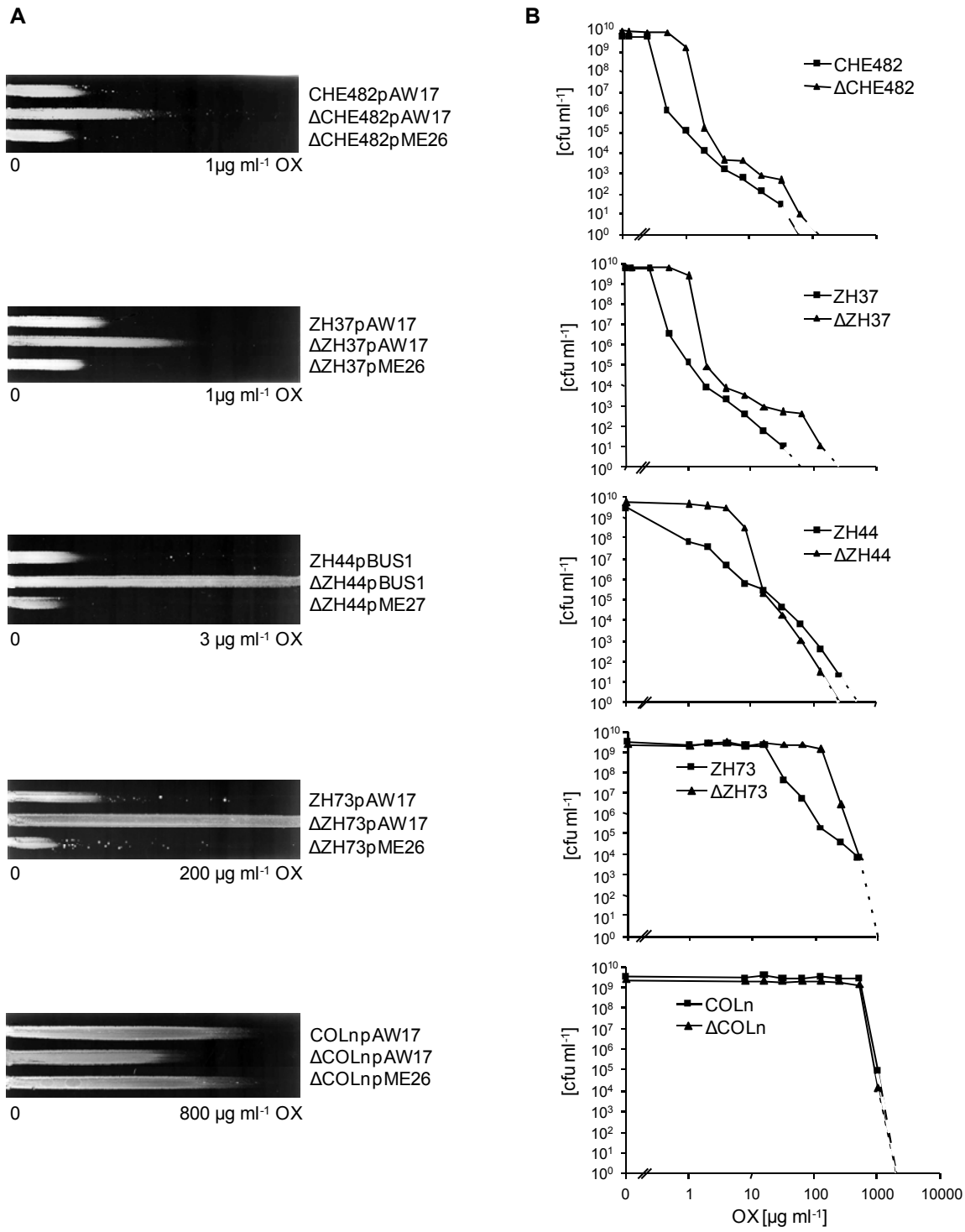

Figure 3.

Effect of SA1665 deletion on oxacillin resistance levels.

A, Growth of MRSA strains, their SA1665 deletion mutants and trans complemented mutants was compared on plates containing the indicated oxacillin gradients. Plates were supplemented with kanamycin 25 µg ml^{-1} or in the case of ZH44 with tetracycline 5 µg ml^{-1} to maintain complementing plasmids.

B, Representative population analysis profiles of MRSA strains CHE482, ZH37, ZH44, ZH73 and COLn and their corresponding mutants. Wildtype strains are indicated by squares and mutants by triangles. x- and y-axis indicate the oxacillin concentrations [µg ml^{-1}] and the colony forming units [cfu ml^{-1}], respectively.

Effect of SA1665 deletion on growth rate. Growth curve analyses showed that deletion of SA1665 reduced the growth rate of all strains, with minimal doubling times of the mutants increased by 3 to 5 minutes (data not shown). The slower growth rates could be connected in some way to the increased oxacillin resistance of most of the mutants (12) although it seems unlikely that the modest changes in resistance levels were enough in themselves to significantly affect growth rates.

Resistance complementation. Primer extension identified two potential SA1665 transcriptional start sites (TSS), one 76-nt and a second 139-nt upstream of the SA1665 ATG start codon (Figure 4).

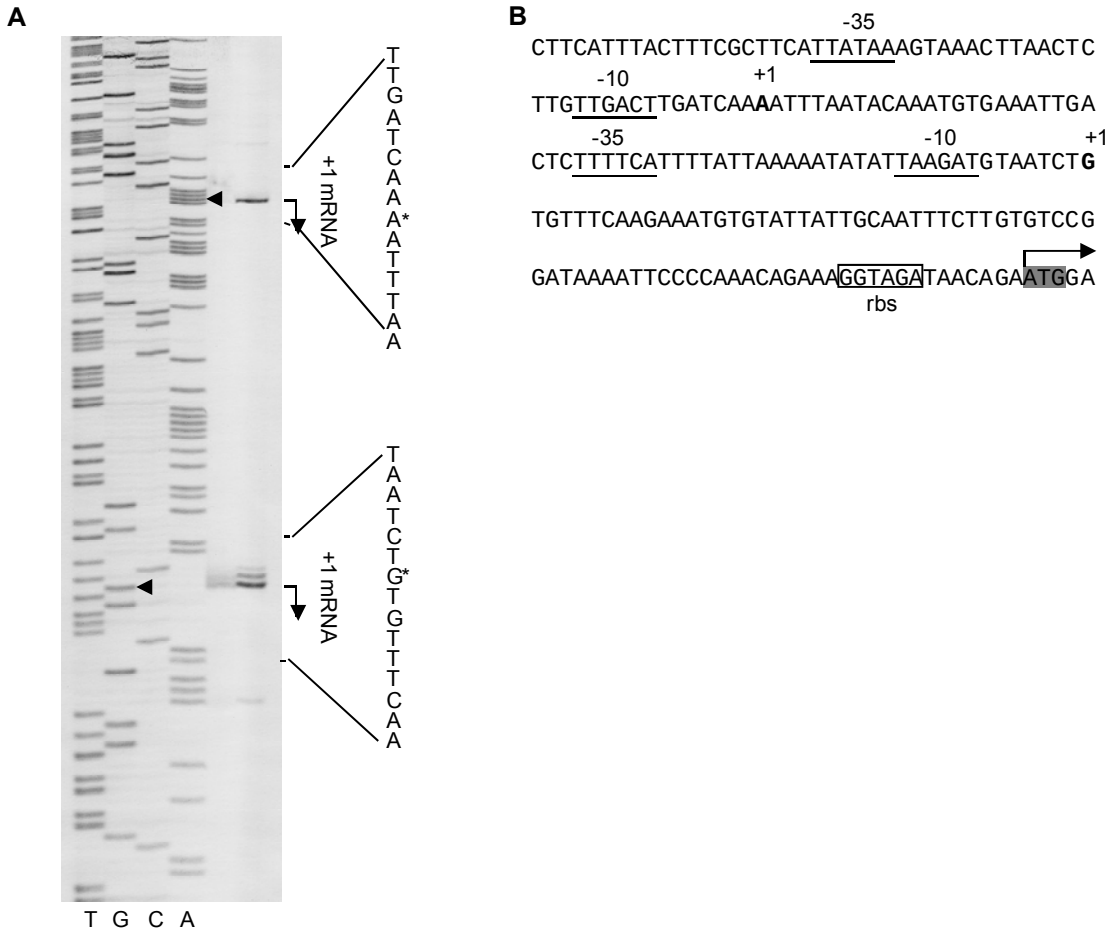

Figure 4.

Primer extension analysis of the transcriptional start site of SA1665.

A, Lanes A, C, G, T show the dideoxy-terminator sequencing ladder. Transcriptional start sites are indicated by arrows and with an asterisk in the partial sequence to the right.

B, Sequence of the SA1665 promoter region. The putative -10 and -35 promoter sequences are underlined, the rbs is framed and the transcriptional start sites +1, are shown in bold. The translational start of SA1665 is highlighted in grey.

Predicted σ^A promoter consensus -10/-35 box sequences were located upstream of both TSS. The -10/-35 sequences (TAAGAT/TTTTCA) for the 76-nt TSS have previously been described in *B. subtilis* (20). The predicted -10/-35 sequences upstream of the 139-nt TSS (TTGACT/TTATAA) were less well conserved, but still shared some similarity with the σ^A consensus (Figure 4).

Plasmids pME26 and pME27 were constructed for complementation of deletion mutants. Both plasmids contained the SA1665 orf along with its own promoter and transcriptional terminator. Strains ΔCHE482, ΔZH37, ΔZH73 and ΔCOLn were complemented with pME26 and ΔZH44 with pME27. Wildtype-like resistance levels were restored in all mutants by introduction of the complementing plasmids (Figure 3A). In the case of ΔZH73pME26, oxacillin resistance was reduced even further than that of wildtype ZH73 containing the empty plasmid pAW17. Small differences in resistance could be determined by oxacillin Etest for CHE482, ZH37 and ZH44 derived strains but not for ZH73 and COLn as MICs were >256 μg ml^{-1} (Table 3).

Table 3. Antibiotic minimal inhibitory concentrations [μg ml^{-1}].

strain	OX
CHE482pAW17	1.5
ΔCHE482pAW17	3
ΔCHE482pME26	1.5
ZH37pAW17	1.5
ΔZH37pAW17	2
ΔZH37pME26	1.5
ZH44pBUS1	96
ΔZH44pBUS1	>256
ΔZH44pME27	4

Abbreviation: OX, oxacillin.

Transcriptional analysis. Northern blot analysis was used to investigate SA1665 expression and the influence of SA1665 deletion on *mecA*, *mecR1* and *blaZ* transcription, as *blaZ* is also under the regulatory control of both MecR1/MecI and BlaR1/BlaI. RNA samples taken from different time points over the growth curve of CHE482 showed that SA1665 was expressed strongly in early exponential phase at OD_{600nm} 0.25 and 0.5 then transcript levels decreased and completely disappeared in stationary phase at OD_{600nm} 4.0 (Figure 5A). In addition to the main transcript of ~0.46 knt a weaker, larger transcript of ~0.6 knt was also visible, especially at later growth stages.

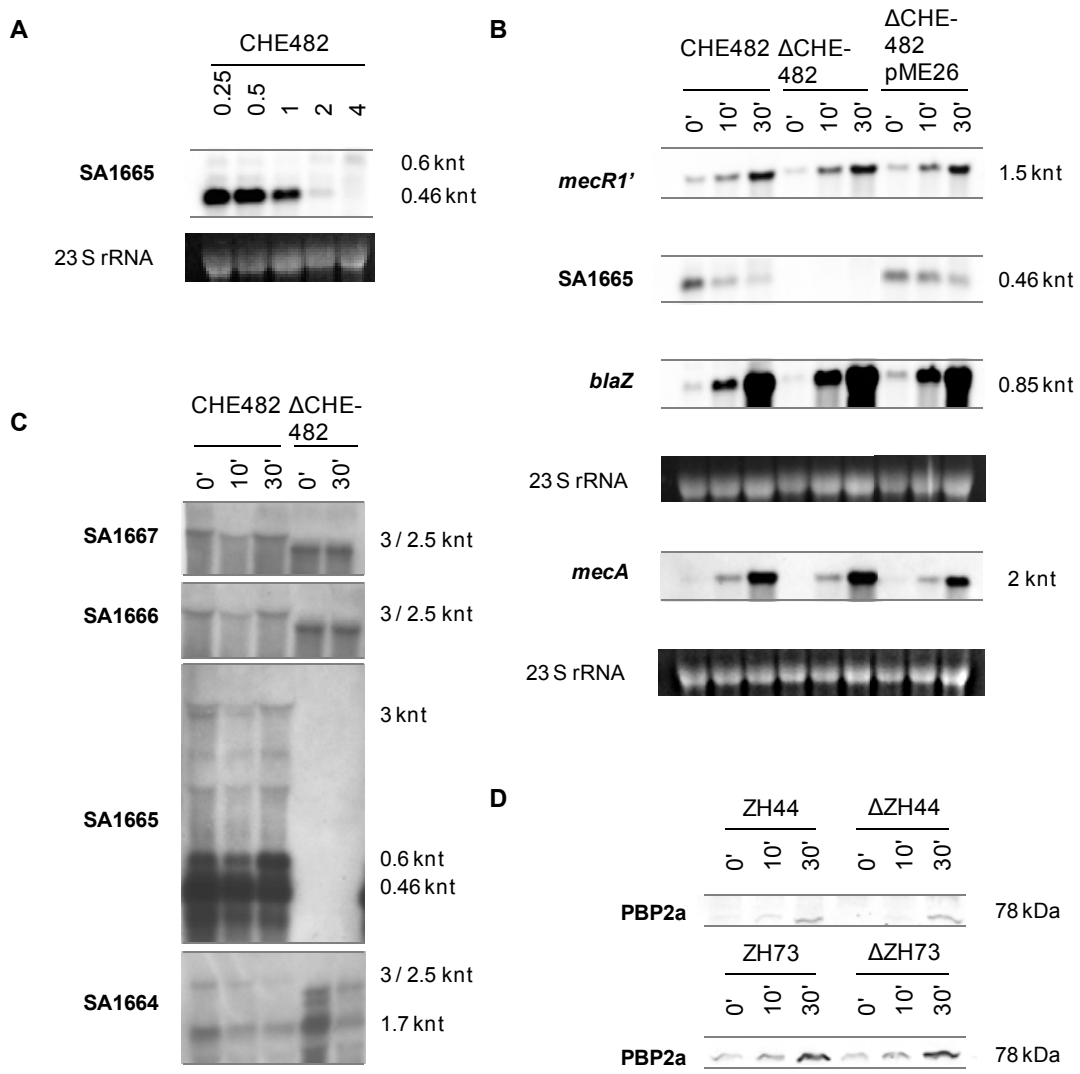

Figure 5.

Northern blot analysis of strains sampled over growth and Northern/Western blot analysis after induction with sub-inhibitory concentrations of cefoxitin. Relevant sampling times or OD_{600nm} values are indicated.

A, Transcription of SA1665 over growth [OD_{600nm}].

B, Transcription of *mecR1*, SA1665, *blaZ* and *mecA* in CHE482, ΔCHE482 and ΔCHE482pME26 before and after induction [min] with cefoxitin 4 µg ml^{-1}. Ethidium bromide stained 23 S rRNA bands are shown as a comparative indication of RNA loading.

C, Transcriptional analysis of CHE482 and its mutant induced [min] with cefoxitin 4 µg ml^{-1}, probed with SA1664, SA1665, SA1666 and SA1667.

D, Western blots of PBP2a protein levels in ZH44, ZH73 and their respective SA1665 deletion mutants, before and after induction [min] with cefoxitin 4 µg ml^{-1}.

Northerns also showed that, as expected, the SA1665 transcript was absent from the deletion mutant, and demonstrated that a wildtype SA1665 transcript was once again present in the complemented mutant ΔCHE482pME26 (Figure 5B). Polar effects were excluded by further Northern blot analyses of neighbouring genes SA1664, SA1666 and SA1667. Interestingly, a weak transcript of about 3 kb was present in all Northerns hybridised with orfs

SA1664-SA1667. This band was absent in the SA1665 mutant when probed with SA1665 and decreased in size to ~2.5 knt when probed with SA1664, SA1666 and SA1667. This suggested that SA1665 was present on several transcripts of different lengths (Figure 5C).
Expression of *mecR1*, *mecA*, *blaZ* and SA1665 was analysed from RNA of uninduced and induced cultures of CHE482, its mutant ΔCHE482 and the complemented strain ΔCHE482pME26. Cells were induced at OD_{600nm} 1.0 with sub-inhibitory concentrations of cefoxitin to relieve BlaI-repression of *mecA* and *blaZ*.
mecR1, although truncated in CHE482, was still transcribed and had the same expression pattern as both *mecA* and *blaZ*, all of which were derepressed over time and had the highest transcript levels after 30 min of induction. In the mutant ΔCHE482, transcription of all three genes was unaffected by SA1665 deletion, indicating that SA1665 has no influence on the expression of *mecA*, *mecR1* or *blaZ*
(Figure 5B).

Western blot analysis. Mutants of ZH44 and ZH73, which had the largest differences in oxacillin resistance levels, were analysed by Western blot analysis to determine if SA1665 affected translation of PBP2a from *mecA*. As indicated in Figure 5D, both pairs of wildtype and mutant strains had the same amounts of PBP2a present both before and after induction with cefoxitin, indicating that SA1665 deletion did not affect translation. Therefore it seems that SA1665 exerts no direct control over *mecA* or PBP2a expression.

Conclusion

β-lactam resistance in MRSA is primarily dependent on the presence of the *mecA* gene, however, resistance levels are generally governed by strain-specific factors including *mecA* regulatory elements and other chromosomal *fem/aux* factors which either enhance or repress the expression of resistance. For instance, the very low-level β-lactam resistance of the Zurich "drug clone" *S. aureus* strain CHE482 was found to be controlled by its genetic background (Ender 2007, accepted), suggesting that it either contained or lacked certain *fem/aux* factors involved in controlling resistance expression. Many of the currently known *fem/aux* factors are directly or indirectly involved in cell wall synthesis and turnover, or envelope biogenesis, however there are still several factors of unknown function.

SA1665, a predicted DNA-binding transcriptional regulator, was found to bind to a DNA fragment containing the *mecA* promoter region and a section of the 5'-*mecA* coding sequence. This protein can be classified as a new *fem/aux* factor, as oxacillin resistance levels were increased in four different MRSA and decreased in one highly resistant MRSA strain, upon SA1665 deletion. However, although this protein binds to the *mecA* promoter/coding sequence it does not appear to directly control *mecA* transcription or induction. It must therefore modulate β-lactam resistance in a *mecA*-independent manner. Its helix-turn-helix DNA-binding transcription factor homology suggests that it controls cellular functions affecting resistance levels.

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3.4 Project 4: Random transposon insertion library of CHE482, a methicillin resistant Staphylococcus aureus (MRSA): Identification of novel fem factors

Abstract

Methicillin resistance in *Staphylococcus aureus* is not only dependent on *mecA* expression and the amount of PBP2a produced but also on chromosomal factors outside the resistance island, SCC*mec* (staphylococcal cassette chromosome *mec*). Chromosomal genes found to influence methicillin resistance have been classified as so called *fem* (factors essential for methicillin resistance) or *aux* (auxiliary genes) factors, but there are still large gaps in our understanding of the genetic mechanisms governing the wide range of different resistance phenotypes, indicating that there are additional, as yet undiscovered *fem/aux* factors. A new transposon-mutant library of CHE482, a very low resistant MRSA (oxacillin MIC 1.5 µg ml^{-1}) with a completely different genetic background to strains used in previous studies, was generated to screen for either elevated or diminished resistance levels. Seventeen new orfs and four previously published orfs were identified by transposon inactivation.

Introduction

Inhibition of proper cell wall biosynthesis by β-lactams, leading to growth arrest and cell lysis, is induced by the acylation and inactivation of the native penicillin binding proteins (PBPs), which are responsible for the crosslinking of peptidoglycan. β-lactams act as substrate analogues of the D-Ala-D-Ala of the peptidoglycan stem peptide (38), which is used by the PBPs in the transpeptidation reaction. In methicillin-resistant *Staphylococcus aureus* (MRSA) an acquired foreign PBP, PBP2a with a lower affinity to β-lactam antibiotics than the native PBPs, sustains cell wall biosynthesis in cooperation with the transglycosylase domain of PBP2, a bifunctional protein with transpeptidase and transglycoslyase activities (30). PBP2a, encoded by *mecA*, is localized on the staphylococcal cassette chromosome *mec* (SCC*mec*), a mobile resistance island integrating into *orfX*, an open reading frame of unknown function (19). *mecA* is either regulated by the divergently transcribed regulatory genes *mecR1/mecI*, coding for a sensor transducer and repressor protein; or, due to the structural and functional similarity of MecR1/MecI with BlaR1/BlaI, by the regulatory elements of the penicillinase gene (27). In so called pre-MRSA, regulated by MecI/MecR1, *mecA* transcription is highly repressed in the absence of an inducer and is induced so slowly by β-lactams that they appear β-lactam sensitive (23).

It is known that PBP2a expression levels do not directly correlate with methicillin resistance levels and that other factors, depending on a strains genetic background, are responsible for determining the strains resistance level (4, 29, 32). Several factors have been identified to be necessary for methicillin resistance expression (4, 6, 11). These so called *fem* (factors essential for methicillin resistance) or *aux* (auxiliary) factors include housekeeping genes involved in cell wall biosynthesis, turnover or regulation (5), protein kinases, ABC transporters, and the catabolite control protein CcpA (11, 35). Inactivation of most *fem/aux* factors do not alter *mecA* expression, except for *murE* coding for a UDP-*N*-acetylmuramyl tripeptide synthetase, which was postulated to either directly or indirectly regulate *mecA* (PBP2a) and *pbpB* expression (14).

A method used to find new factors involved in methicillin resistance is the creation of a random transposon insertion library in an MRSA strain and subsequent screening for altered drug resistance levels. Mutant libraries were previously generated in NCTC8325-*mec* and COL backgrounds, both of which are highly resistant strains, using the transposon Tn*551*. In this study an extremely low level oxacillin resistant strain CHE482, spreading in the area of Zurich, Switzerland was chosen to generate a random transposon library, allowing the selection of mutants with either increased or decreased β-lactam resistance. A higher yield of mutants, especially those identifying novel genes was anticipated here because we used the more random and highly efficient mariner transposon, *bursa aurealis* (2). The new library generated and analysed here expands the list of factors which affect methicillin resistance.

Materials and methods

Bacterial strains and growth conditions. Strain CHE482, expressing low level oxacillin resistance, fusidic acid and trimethoprim resistance, was used to generate a random transposon insertion library (12, 31). The plasmids used were pFA545, carrying a transposase and tetracycline and ampicillin resistance genes and pBURSA containing the transposon *bursa aurealis* which encodes erythromycin resistance (2). The strains were grown in/on Luria Bertani broth/agar (LB, Difco) or tryptic soy agar (TSA, Difco) at 37°C or 43°C. CHE482 carrying pBURSA was grown at 30°C due to the temperature sensitive origin of replication of pBURSA. Media were supplemented with 5 µg ml^{-1} tetracycline or 10 µg ml^{-1} erythromycin when required.

Transposon mutagenesis. CHE482 was sequentially transformed by direct electroporation with pFA545 and pBURSA. Direct electroporation was done as described in (20), whereby cells were mixed with 500 ng of plasmid DNA, incubated for 10 min on ice and electroporated using following settings: 25 µF, 2.0 kV and 100 Ω. Single colonies, growing on selective plates, were resuspended in 200 µl of prewarmed (43°C) dH_2O. Twenty µl of a 10^{-1} and 100 µl of a 10^{-2} dilution were plated on prewarmed TSA plates supplemented with 10 µg ml^{-1} erythromycin. Colonies grown for 2 days at 43°C were picked into microtiter dishes containing LB (Difco) and were grown overnight at 37°C. To screen for higher and lower resistance, all wells were replica plated onto LB agar plates containing 0, 5 or 16 µg ml^{-1} of cefoxitin.

Southern blotting. Confirmation of integration of *bursa aurealis* was done by Southern blot as described elsewhere (1). Hybridisation was done using an *ermB*-DIG labelled probe, generated with primers ermB-fwd and ermB-rev (5'-CGAAATTGGAACAGGTAAAG-3'/5'-TTTATCTGGAACATCTGTGG-3') using a PCR DIG probe synthesis kit (Roche).

Susceptibility testing. Qualitative resistance comparisons were done by swabbing cells resuspended to a McFarland density of 0.5 on LB agar plates containing an increasing gradient of cefoxitin (0-8 µg ml^{-1}). Plates were incubated at 35°C for 24 h.

Phenotype confirmation. Phage 85α lysates of the CHE482 mutants were used to back transduce the mutations into the original wildtype CHE482. All back transductants were again analysed on gradient plates.

Identification of transposon integration sites. Transposon integration sites of mutants with different resistance phenotypes were determined by inverse PCR using primers Martn-F

and Martn-ermR, and sequenced with Martn-F (2) on an ABI Prism 310 genetic analyzer (Applied Biosystems) using the ABI PRISM BigDye Terminator Cycle sequencing reaction kit (U.S. Biochemicals). Blast searches were done using the NCBI server (http://www.ncbi.nlm.nih.gov/BLAST/) and sequence alignments were performed with MultAlin (9).

Results and Discussion

Transposon library. The very low level β-lactam resistant strain CHE482 belongs to clonal complex 45 and sequence type ST45, has a cefoxitin MIC of 12 µg ml^{-1}, carries a mecB complex (IS*1272-ΔmecR1-mecA*) and a penicillinase plasmid (12). This strain was transformed sequentially with pFA545 (encoding a transposase) and pBURSA (carrying the transposon), and selected for integration of *bursa aurealis* into the genome (2). A library comprising 8064 mutants was generated, which assuming random integration and an average orf size of ~ 1 kb, every orf within the staphylococcal genome should be interrupted approximately 3 times; based on data from the reference strain N315 which has a genome size of 2.8 Mbp and 2669 open reading frames (orfs). Subtracting the number of essential orfs, that cannot be disrupted, further increased the probability that most non-essential genes were interrupted. Transposon integration and randomness were confirmed in a selection of mutants by Southern hybridisation using an *ermB* probe to demonstrate integration in different sized restriction fragments.

Mutant screening and phenotypic confirmation. The library, grown in a 96 well microtiter dish over night, was replica plated onto agar plates containing 0, 5 or 16 µg ml^{-1} of cefoxitin, respectively, to screen for mutants with potentially lower or higher cefoxitin resistance, respectively. Two hundred and nine mutants expressing a putatively different phenotype were further analysed for growth on plates containing a gradient of 0 to 8 µg ml^{-1} cefoxitin, to directly compare resistance differences with their parent CHE482. This method enabled discrimination of small differences in resistance levels. Only 51 (24.4%) mutants with lower and 14 (6.7%) with higher resistance were confirmed to have differences in resistance and then checked for linkage of the phenotype to the *bursa aurealis* insert by back transduction into CHE482. Linkage could be confirmed in 45 mutants by back transduction (Table 1). Eight mutants, with only slight differences, could not be clearly discriminated from the wildtype and their small differences observed immediately after back transduction were generally lost upon further passaging, suggesting selection of adapted variants (Table 2). In general, mutants produced single colonies at higher antibiotic concentrations on cefoxitin gradient plates indicating the emergence of compensatory mutants. Transduction of the *bursa aurealis* insert of these 8 putative *fem/aux* factors into 2 highly resistant MRSA strains BB270 and COL is ongoing to clarify their influence on methicillin resistance. Preliminary results showed altered resistance levels and therefore evidence for their function as *fem/aux* factors.

The number of mutants expressing higher resistance was strongly reduced after back transduction. Linkage of the insert to higher resistance could be confirmed in only three out

of the 14 mutants. Presumably, since spontaneous selection of highly resistant variants occurred on the screening plates, independently of the insertion.

Integration sites. The insertion sites of *bursa aurealis* were identified by iPCR and subsequent sequencing. Integration sites and their distance to the next start or stop codon and their corresponding cefoxitin phenotype on gradient plates are shown in Figure 1. Since integration of *bursa aurealis* results in a TA duplication, the integration sites indicated are correct within ± 2 bp (2). The putative gene functions and cellular roles, as indicated in the Tigr database (www.tigr.org), are listed in Tables 1 and 2.

bursa aurealis was shown not to have a strong bias towards insertion in certain "hotspot" regions, unlike Tn*551* and Tn*917* (2, 3, 10). Multiple identical integration sites identified within a single gene were counted as one event since proliferation of a single mutant prior to the screening procedure could not be ruled out.

Looking at the interruption efficiency of *bursa aurealis* in Newman, reported by Bae et al. (2), indicated that a higher number of mutants, approximately twice the number used here, would have been necessary to hit all non-essential genes. Nevertheless the 45 mutants expressing an altered resistance phenotype identified here could be attributed to the inactivation of 21 different orfs, 19 of which generated reduced resistance and only two of which increased resistance. The cellular roles of these 21 orfs are mainly unclassified or are associated with the cell envelope and transport-binding proteins (Table 3). Of the 21 orfs found in this study only four orfs were identical to previously published factors known to influence methicillin resistance.

Published factors involved in methicillin resistance. *bursa aurealis* inserts were identified in *mecA* (Figure 1, Nr. 1), *blaR1* (Figure 1, Nr. 42-45) and *alr* (Figure 1, Nr. 33-35), three genes known to be required for methicillin resistance: *mecA* codes for PBP2a, whose transpeptidase domain is able to sustain cell wall crosslinking in the presence of β-lactam antibiotics in cooperation with the transglycosylase domain of PBP2. Its low affinity to all β-lactam antibiotics allows it to avoid acylation and consequent inactivation, which confers resistance to β-lactam antibiotics (16, 30).

The interruption of the β-lactam sensor transducer, BlaR1, leads to continuous repression of *mecA* transcription as it can no longer mediate cleavage of the BlaI repressor, virtually abolishing *mecA* transcription and thus the synthesis of PBP2a, the prerequisite of methicillin resistance (33). Four separate mutants with decreased resistance were found to have insertions in BlaR1.

The alanine racemase, encoded by *alr*, interrupted by *bursa aurealis* at 3 different sites, is responsible for the conversion of L-alanine to D-alanine. D-alanine is incorporated in the peptidoglycan stem peptide and is involved in the crosslinking reaction in bacterial cell wall synthesis. Other factors involved in cell wall precursor synthesis have also been shown to decrease resistance when interrupted (5, 21).
The fourth previously identified orf is SA1063, a protein kinase, found both here and in an earlier study using a COL transposon mutant library (11) to reduce β-lactam resistance. No further characterisation of this gene and its function has been done so far.

Novel factors. The remaining 17 orfs identified are of unknown function and whether they directly or indirectly influence methicillin resistance still has to be evaluated. Transposon insertions may also affect neighbouring genes that might be organised in the same operon, so polar up- or down-stream effects, in the case of SA0461/SA0462/SA0463 and SA1563/ SA1564, can not be excluded. Information about the putative function of these 17 orfs is very scarce.

Factors decreasing resistance. Some information on putative function of four of these 17 genes may be deduced from sequence homologies to published genes.
SA0461: The *mfd* gene (Figure 1, 4-7), coding for a transcription repair coupling factor, was described to be regulated by MgrA a major global regulator, which is involved in regulating virulence and antibiotic resistance (25).
SA0462: The interrupted *rfbX* gene (Wzx) (Figure 1, Nr. 8-16) in Gram-negatives, codes for a predicted flippase with 12 transmembrane domains, which may be responsible for the flipping of the lipid-bound O-polysaccharide subunits across the membrane to the outside, where polymerisation of these subunits takes place. Its function in *S. aureus* and influence on methicillin resistance has not yet been reported (24, 34).
SA0809: *mnhE* (Figure 1, Nr. 19) forms part of an operon consisting of 7 orfs (*mnhA-mnhG*), encoding an Na^+/H^+ antiporter. Interruption of *mnhE* might have an influence on the internal pH, the export of Na^+ and Li^+, the cell's internal volume or the electrochemical potential across the membrane (17).
SA1463: *secDF* (Figure 1, Nr. 25) codes for a protein with 12 putative transmembrane domains and two extracytoplasmic loops, which has been shown to form part of a sec translocation pathway in *Bacillus subtillis*. Protein transport was found to be just slightly affected by its deletion. SecDF is postulated to improve protein secretion capacities (7, 36).

Factors increasing resistance. Integration of *bursa aurealis* into SA0756/*aroD*, a hypothetical protein similar to 3-dehydroquinate dehydratase (Figure 1, Nr. 18), and into the promoter/operator region of SA1054 (Figure 1, Nr. 21-22), coding for *dfp*, a panthothenate metabolism flavoprotein homolog, increased resistance slightly. Interruption of *aroD*, which together with phosphoenol pyruvate converts D-erythrose 4-phosphate to chorismate, used in ubiquinone, folate and siderophore biosynthesis, resulted in strongly impaired growth (UniprotKB/Swissprot/KEGG database). SA1054/*dfp* is involved in pantothenate and CoA biosynthesis, where it catalyses the synthesis of the CoA precursor 4'-phosphopentetheine. Due to an inverse relationship between resistance levels and growth rate (13), the slightly increased resistance might be partially due to the slow growth. Secondly, since genes of the coenzyme A biosynthetic pathway are essential, the *bursa aurealis* integration into the *dfp* promoter/operator region suggests that *dfp* expression was reduced but not abolished (22).

Phenotypes compensated upon subculturing. The resistance phenotypes of the eight interrupted orfs shown in Table 2 were lost upon subculturing. Whether these mutations were compensated by another pathway or gene function has still to be investigated.

One of these genes *glpD* (Figure 1, Nr. 48), encoding glycerol-3-phosphate dehydrogenase, is involved in glycolysis, converting glycerol-3-phosphate to dihydroxyacetone phosphate. A possible cre-site (catabolite-responsive element) recognised by CcpA (catabolite control protein) was found in the initial sequence of the GlpD coding region of *B. subtilis* but not in *S. aureus* (personal communication K. Seidl) (11, 15, 35, 28).

trpE (Figure 1, Nr. 49) produces the catalytically functional subunit of anthranilate synthase, the first enzyme of the tryptophan synthesis pathway converting chorismic acid to anthranilic acid (18, 40).

ribA (Figure 1, Nr. 50) is a gene in the *rib* operon consisting of *ribG-ribB-ribA-ribH* catalysing conversion of ribulose-5-phosphate to riboflavin (26).

citT (yflQ), (Figure 1, Nr. 51) together with *citS*, encodes a two-component system. CitT controls transcriptional expression of CitM, a Mg^{2+}-citrate transporter (39).

Region of special interest. Inactivation of SA1661, a putative membrane protein, reduced methicillin resistance levels (Figure 1, Nr. 31-32). The converse effect was observed when SA1665, approximately 3.6-kb upstream of SA1661, was deleted. SA1665 is a putative DNA-binding protein and transcriptional regulator that binds to the *mecA* coding sequence (see section 3.3). Adjacent to SA1665 is a two component system encoded by *yhcS* (SA1666) and *yhcR* (SA1667) that has been shown to influence cell wall biosynthesis, as fosfomycin resistance was reduced upon *yhcS* antisense RNA expression (37). Therefore this gene cluster, composed of 3 operons and an additional single transcriptional unit (putative operons were identified using http://www.softberry.com), contains several regulatory elements/genes (Figure 2) affecting methicillin resistance levels or cell wall biosynthesis and is therefore an interesting target for further studies.

Conclusion

The staphylococcal genome shows high variability with a sequence identity of 94.7 % to 99.7 % between strains N315, Mu50 and MW2 (2). Therefore, some factors affecting antibiotic resistance levels might be present or active in some strains but not in others.

In this study, certain genes affecting resistance levels might have been missed due to the low initial methicillin resistance in CHE482, limiting the screening for reduced resistance. The number and selection of *fem/aux* factors found depends on the genetic background of a strain, on the transposon, and the selection conditions used. A supplementary factor is that the β-lactam antibiotic used, cefoxitin, a cephamycin with high affinity to PBP4 (8, 41), used in this study might select for different genetic determinants than methicillin or other β-lactams. Further investigation of these interrupted genes is ongoing. This involves transducing the insertions into MRSA of different genetic background expressing high homogeneous (COL) and high heterogeneous (BB270) resistance, to confirm their relevance for methicillin resistance. Analysis of direct correlations between *mecA* expression and the interruption of the identified *fem/aux* factors is being done by Northern blot or RT-PCR.

The generation of a transposon library is an interesting tool to identify new *fem/aux* factors, which are not only involved in cell wall biosynthesis but also in cell metabolism. The main fraction of factors affecting methicillin resistance levels are genes of unknown function, requiring further characterisation and functional analysis. So far a large number of genes have been shown to affect methicillin resistance (11), and this number is still expected to increase due to the high variability found in the *S. aureus* genus. Therefore, while the mec complex is essential for methicillin resistance, resistance still depends on and is controlled by other chromosomal determinants (32). A lot of effort is still necessary to identify all *fem/aux* factors and to determine their relevance in methicillin resistance. Genetic factors responsible for the extremely low-level resistance of the drug clone are still unknown and may not be able to be identified by mutagenesis.

Table 1. Mutants of CHE482 altered resistance phenotype. Orf numbers are from N315 (SA) , pN315 (SAP), MRSA252 (SAR).

Nr.[1]	Coord.[2]	Strain nr.	Res. level[3]	Gene/ promoter	Orf number	Putative gene function	Tigr cellular role category
1	38H11	ME333	lower	*mecA*	SA0038	Penicillin binding protein 2 A, PBP2A	Cell envelope: Biosynthesis and degradation of murein sacculus and peptidoglycan
2	59G3	ME311	lower	SA0370 promoter	SA0370	Putative DNA binding protein	Hypothetical proteins (Hyp. Prot.): Conserved
3	5A1	ME291	lower		SA0423	Hyp. prot., similar to autolysin (N-acetylmuramoyl-L-alanine amidase)	Unclassified: Role category not yet assigned
4	23F9	ME338	lower				
5	40D11	ME320	lower	*mfd*	SA0461	Transcription repair coupling factor	DNA metabolism: DNA replication, recombination, and repair
6	34C9	ME312	lower				
7	59F9	ME339	lower				
8	23C9	ME328	lower				
9	67A7	ME299	lower				
10	67A10	ME293	lower	*rfbX*	SA0462	Membrane protein involved in the export of O-antigen and teichoic acid; similar to low temperature requirement B protein	Cell envelope: Biosynthesis and degradation of surface polysaccharides and lipopolysaccharides
11	67B12	ME301	lower				
12	66G5	ME331	lower				

Table 1 continued.

Nr.[1]	Coord.[2]	Strain nr.	Res. level[3]	Gene/ promoter	Orf number	Putative gene function	Tigr cellular role category
13	68EF12	ME313	lower				
14	68G12	ME325	lower	*rfbX*	SA0462	Membrane protein involved in the export of O-antigen and teichoic acid; similar to low temperature requirement B protein	Cell envelope: Biosynthesis and degradation of surface polysaccharides and lipopolysaccharides
15	67A1	ME332	lower				
16	68EF12-2	ME314	lower				
17	34G7	ME315	lower		SA0463	Putative tetrapyrrole (Corrin/Porphyrin) methylase	Unknown function: Enzymes of unknown specificity
18	37D8	ME300	higher		SA0756	Hyp. prot., similar to 3-dehydroquinate dehydratase	Amino acid biosynthesis: Aromatic amino acid family
19	57D3	ME298	lower	*mnhE*	SA0809	Multisubunit Na+/H+ antiporter, MnhE subunit	Transport and binding proteins: Cations and iron carrying compounds
20	63 E1	ME294	lower		SA0931	Putative membrane protein	Cell envelope: Other
21	26G9	ME281	higher	*dfp* promoter	SA1054	Pantothenate metabolism flavoprotein homolog	Biosynthesis of cofactors, prosthetic groups, and carriers: Pantothenate and coenzyme A
22	26H11	ME285	higher				
23	80G8	ME304	lower		SA1063	Protein kinase	Energy metabolism: Electron transport; Regulatory functions: Other; Protein synthesis: Ribosomal proteins: synthesis and modification
24	29B8	ME318	lower				

Table 1 continued.

Nr.[1]	Coord.[2]	Strain nr.	Res. level[3]	Gene/ promoter	Orf number	Putative gene function	Tigr cellular role category
25	17H3	ME305	lower	*secDF*	SA1463	Protein-export membrane protein SecDF	Protein fate: Protein and peptide secretion and trafficking
26	10G10	ME308	lower		SA1501	Conserved hyp. prot.	DNA metabolism: DNA replication, recombination, and repair
27	16B11	ME289	lower		SA1563	Phenylalanyl-tRNA synthetase (beta subunit) homolog	Protein synthesis: tRNA aminoacylation
28	26G1	ME340	lower	SA1564 promoter	SA1564	Conserved hyp. prot.	Hyp. Prot.: Conserved
29	41A11	ME310	lower				
30	46F6	ME316	lower				
31	14D8	ME326	lower		SA1661	Putative membrane protein	Hyp. prot.: Conserved
32	21B9	ME317	lower				
33	20A1	ME292	lower	*alr*	SA1874	Alanine racemase	Cell envelope: Biosynthesis and degradation of murein sacculus and peptidoglycan
34	10H8	ME322	lower				
35	79H9	ME330	lower				

Table 1 continued.

Nr.[1]	Coord.[2]	Strain nr.	Res. level[3]	Gene/ promoter	Orf number	Putative gene function	Tigr cellular role category
36	18C3	ME302	lower				
37	13F9	ME321	lower	*corA*	SA2137	Hyp. prot. similar to divalent cation; Mg^{2+} and Co^{2+} transporters	Transport and binding proteins: Unknown substrate; Transport and binding proteins: Amino acids, peptides and amines
38	3D6	ME280	slightly lower				
39	59 E4	ME309	lower				
40	21C8	ME327	lower	*manB?*	SA2279	Hyp. prot., similar to phosphomannomutase	Energy metabolism: Sugars
41	13F12	ME296	lower	*cycA (dagA)*	SAR1775	Putative D-serine/D-alanine/glycine transporter	Transport and binding proteins: Amino acids, peptides and amines
42	30A7	ME282	lower				
43	79F4	ME334	lower	*blaR1*	SAP011	Bla regulator protein BlaR1, pN315 β-lactamase plasmid	Regulatory functions: Other
44	5H6	ME306	lower				
45	22F10	ME307	lower				

[1] Identical integration sites are indicated by bold numbers.

[2] Coordinates of the microtiter dishes comprising the mutant collection.

[3] Resistance level compared to parent strain.

Table 2. Mutants of CHE482 initially exhibiting a resistance phenotype. Orf numbers are from N315 (SA) , pN315 (SAP), NCTC8325 (SAOUHSC).

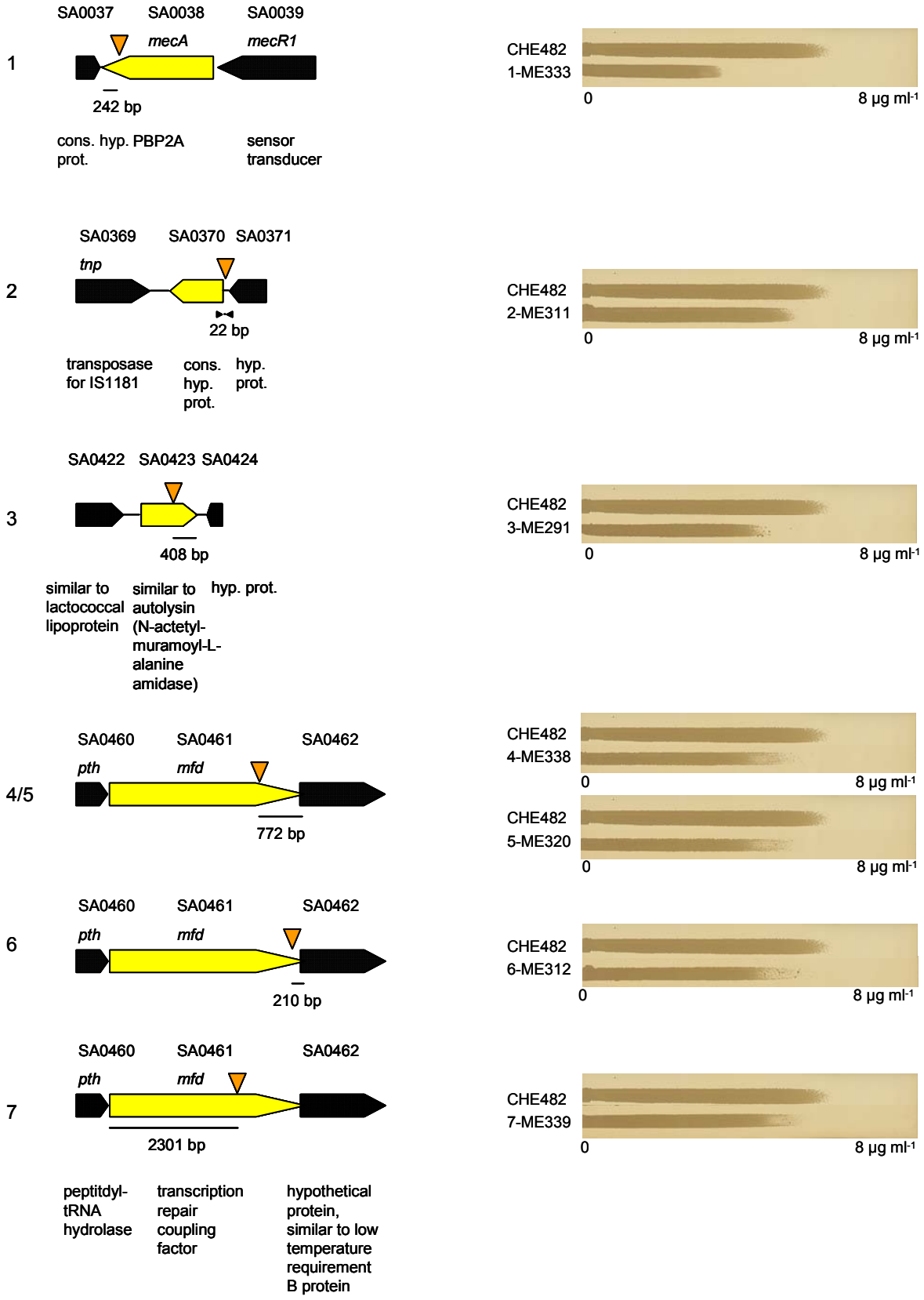
Nr.[1]	Coord.[2]	Strain nr.	Res. level[3]	Gene/ promoter	Orf number	Putative gene function	Tigr cellular role category
46	MA11	ME343	no difference		SA0241	Hyp. prot., similar to 4-diphosphocytidyl-2C-methyl-D-erythritol synthase	Unclassified: Role category not yet assigned
47	70G8	ME358	no difference		SA0836	Hyp. prot., similar to transcription regulator LysR family	Regulatory functions: DNA interactions
48	35H12	ME297	no difference	*glpD*	SA1142	Aerobic glycerol-3-phophate dehydrogenase	Energy metabolism: Other
49	74D3	ME344	no difference	*trpE*	SA1199	Hyp. prot., similar to anthranilate synthase component I	Amino acid biosynthesis: Aromatic amino acid family
50	64 E10	ME341	no difference	*ribA*	SA1587	GTP cyclohydrolase II; riboflavin biosynthesis protein	Biosynthesis of cofactors, prosthetic groups, and carriers: Riboflavin, FMN, and FAD
51	31 E10	ME286	no difference	*citT*	SA2486	Di- and tricarboxylate transporters, 2-oxoglutarate/malate translocator homolog	Transport and binding proteins: Anions
52	63C6	ME287	no difference		SAOUHSC02047	Phage putative head morphogenesis protein, SPP1, gp7 family domain protein	Mobile and extrachromosomal element functions: Prophage functions
53	63C10	ME357	no difference				
54	19F3	ME319	no difference	SAP031 promoter	SAP031	Hyp. prot., pN315 β-lactamase plasmid	

[1] Identical integration sites are indicated by bold numbers.

[2] Coordinates of the microtiter dishes comprising the mutant collection.

[3] Resistance level compared to parent strain.

Figure 1.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SA0037
SA0038
SA0039
mecA
mecR1
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cons. hyp. prot.
PBP2A
sensor transducer
CHE482
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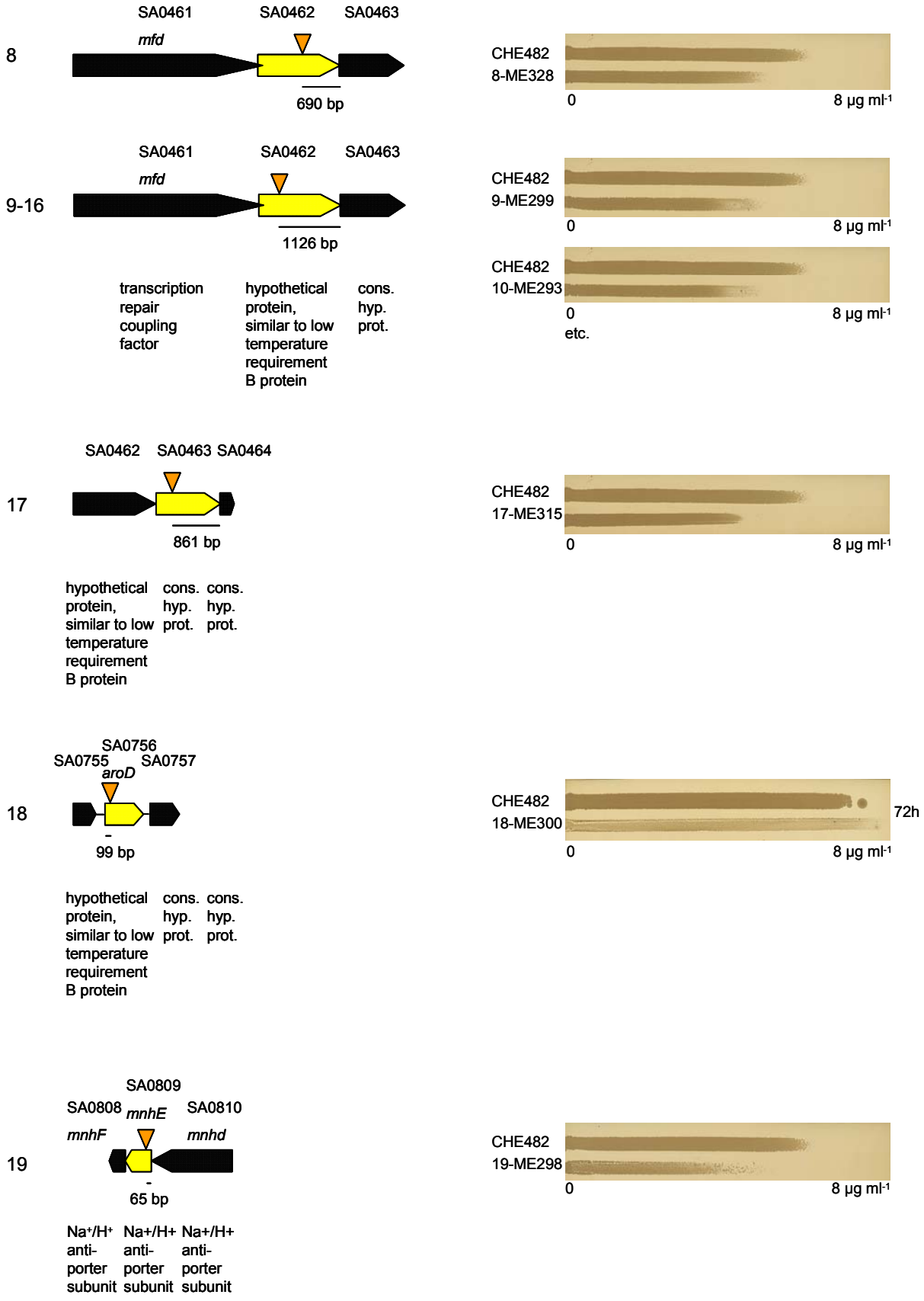
2
SA0369
SA0370
SA0371
tnp
22 bp
transposase for IS1181
cons. hyp. prot.
hyp. prot.
CHE482
2-ME311
0
8 µg ml-1
3
SA0422
SA0423
SA0424
408 bp
similar to lactococcal lipoprotein
similar to autolysin (N-actetyl-muramoyl-L-alanine amidase)
hyp. prot.
CHE482
3-ME291
0
8 µg ml-1
4/5
SA0460
SA0461
SA0462
pth
mfd
772 bp
CHE482
4-ME338
0
8 µg ml-1
CHE482
5-ME320
0
8 µg ml-1
6
SA0460
SA0461
SA0462
pth
mfd
210 bp
CHE482
6-ME312
0
8 µg ml-1
7
SA0460
SA0461
SA0462
pth
mfd
2301 bp
peptitdyl-tRNA hydrolase
transcription repair coupling factor
hypothetical protein, similar to low temperature requirement B protein
CHE482
7-ME339
0
8 µg ml-1

Figure 1 continued.



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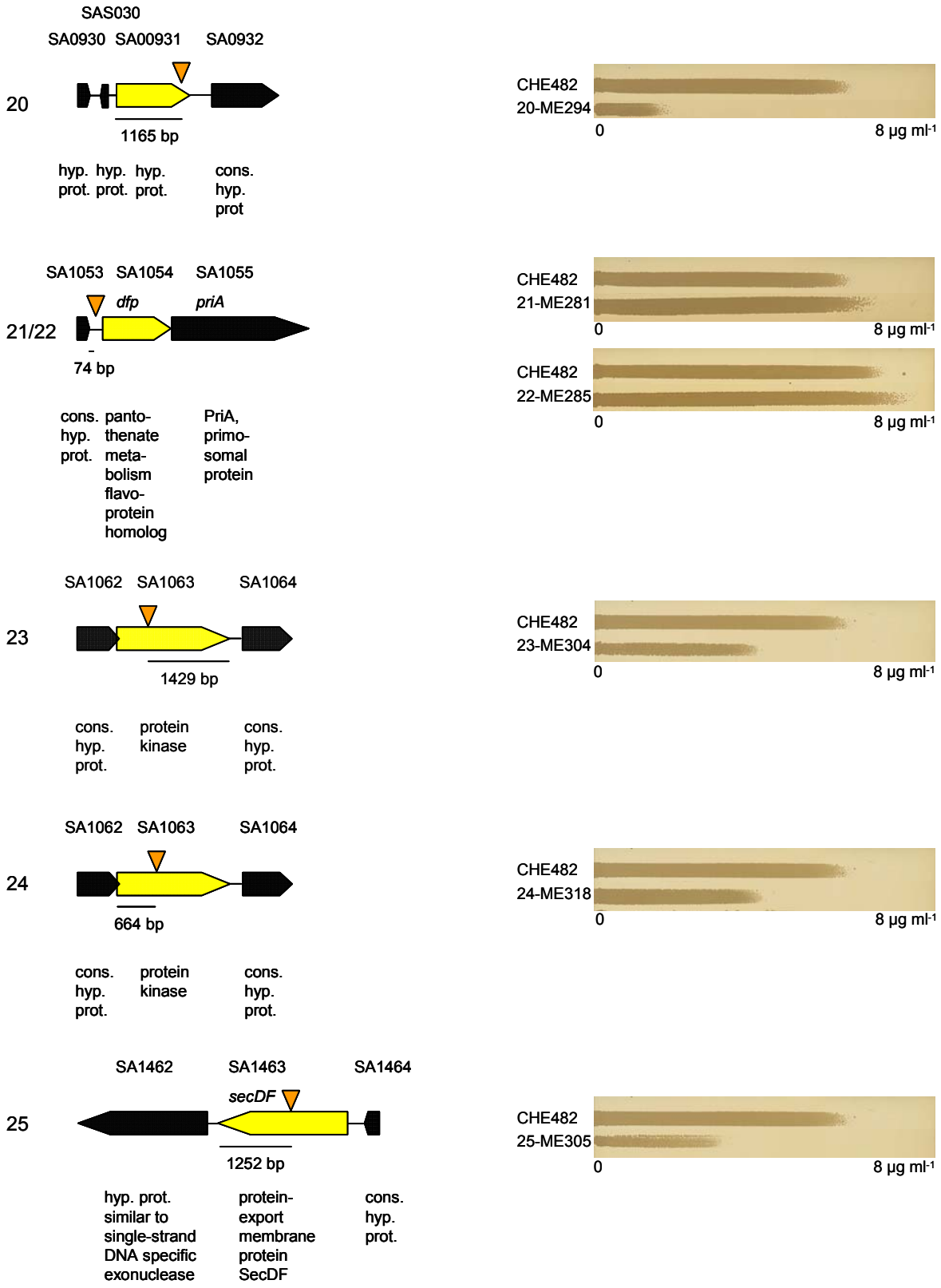

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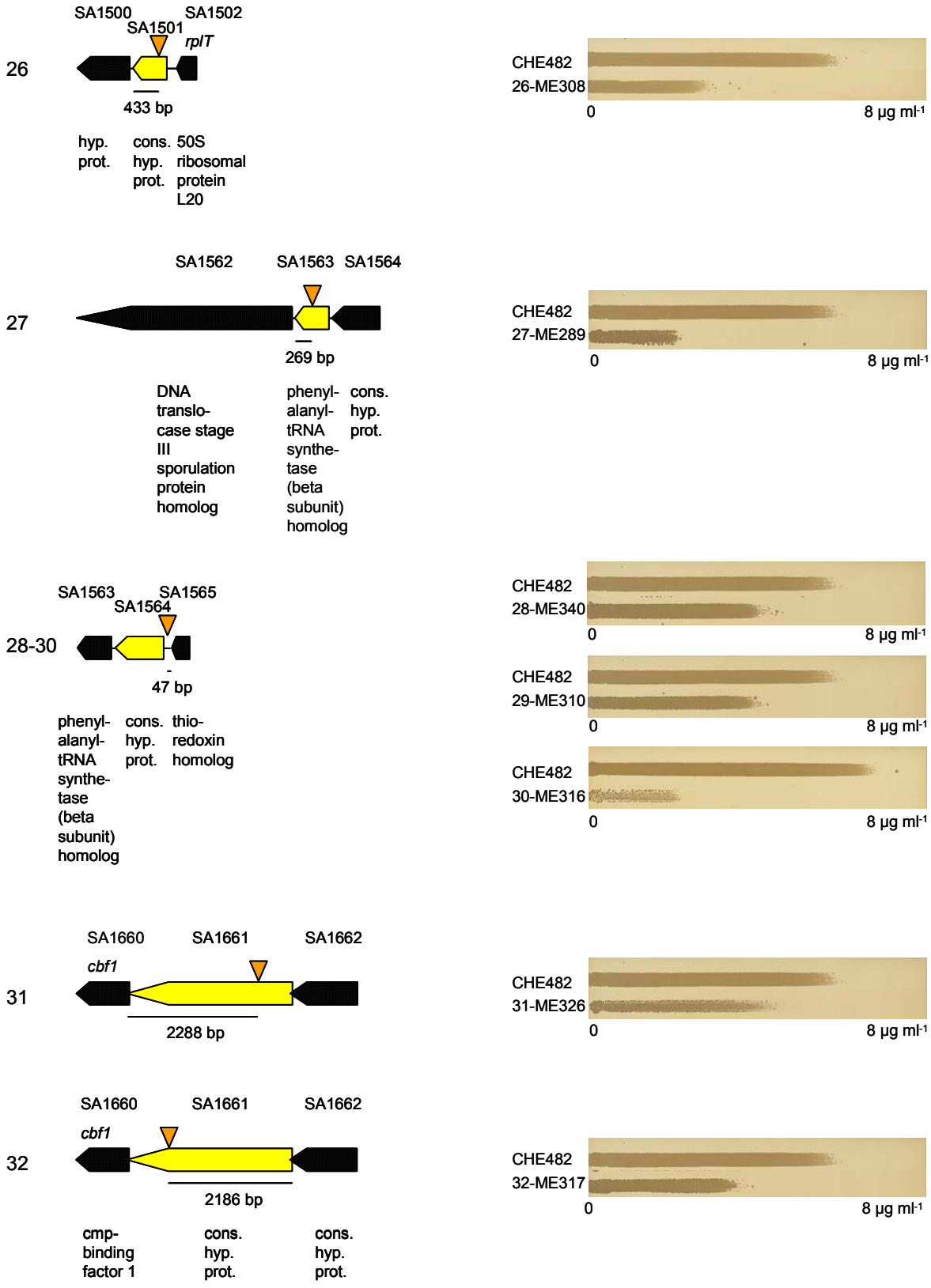

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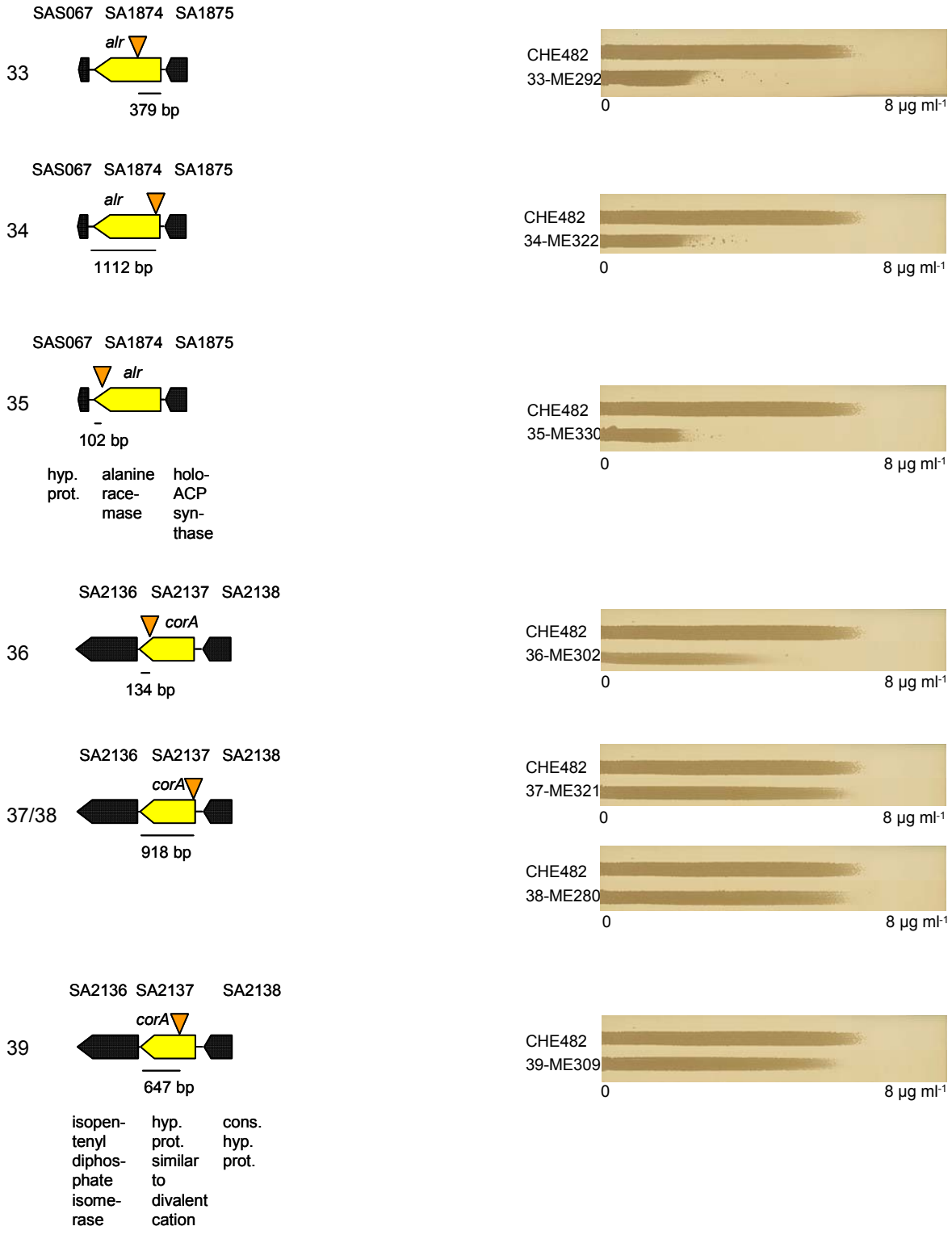

Figure 1 continued.

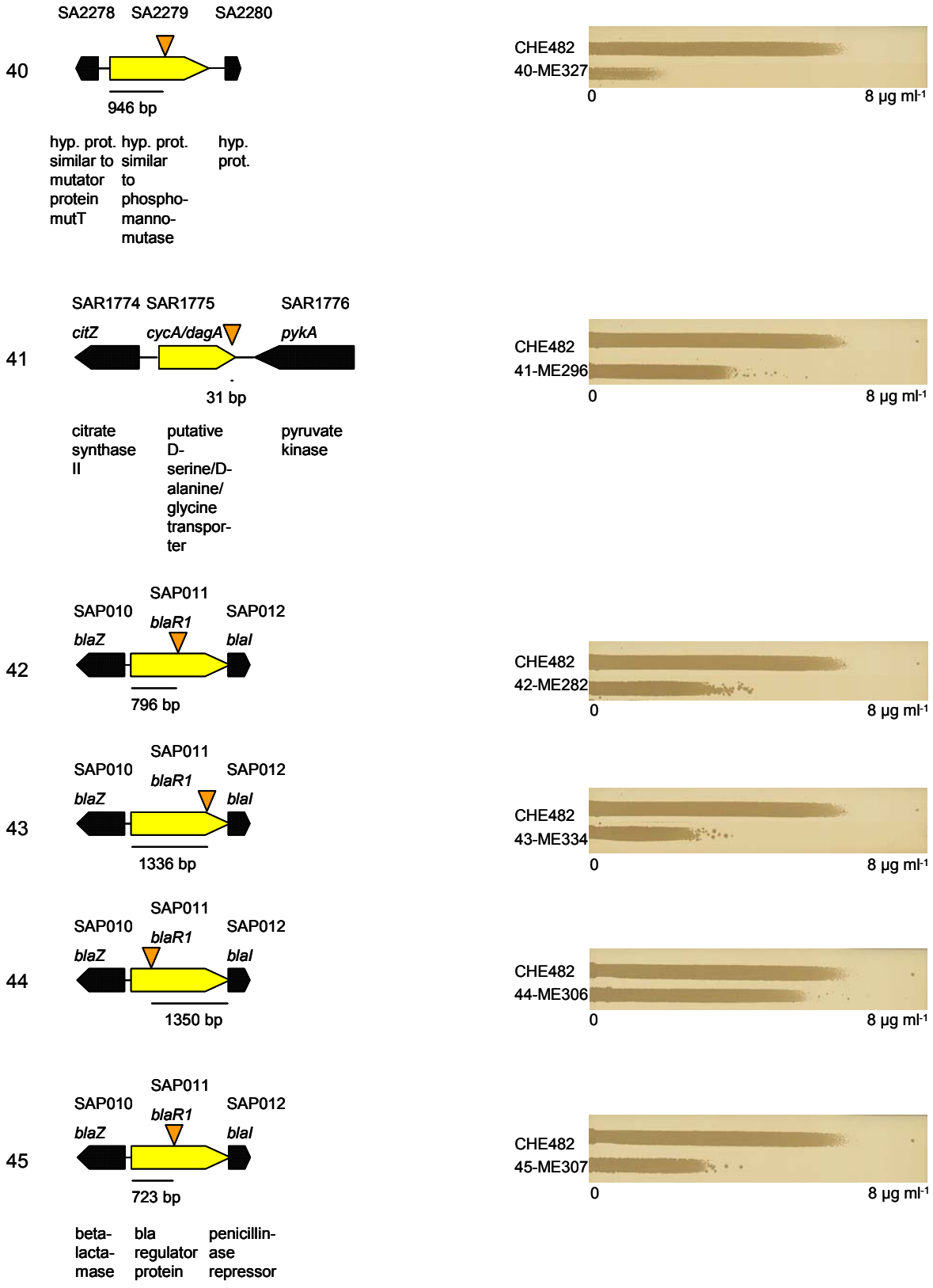

Figure 1 continued.

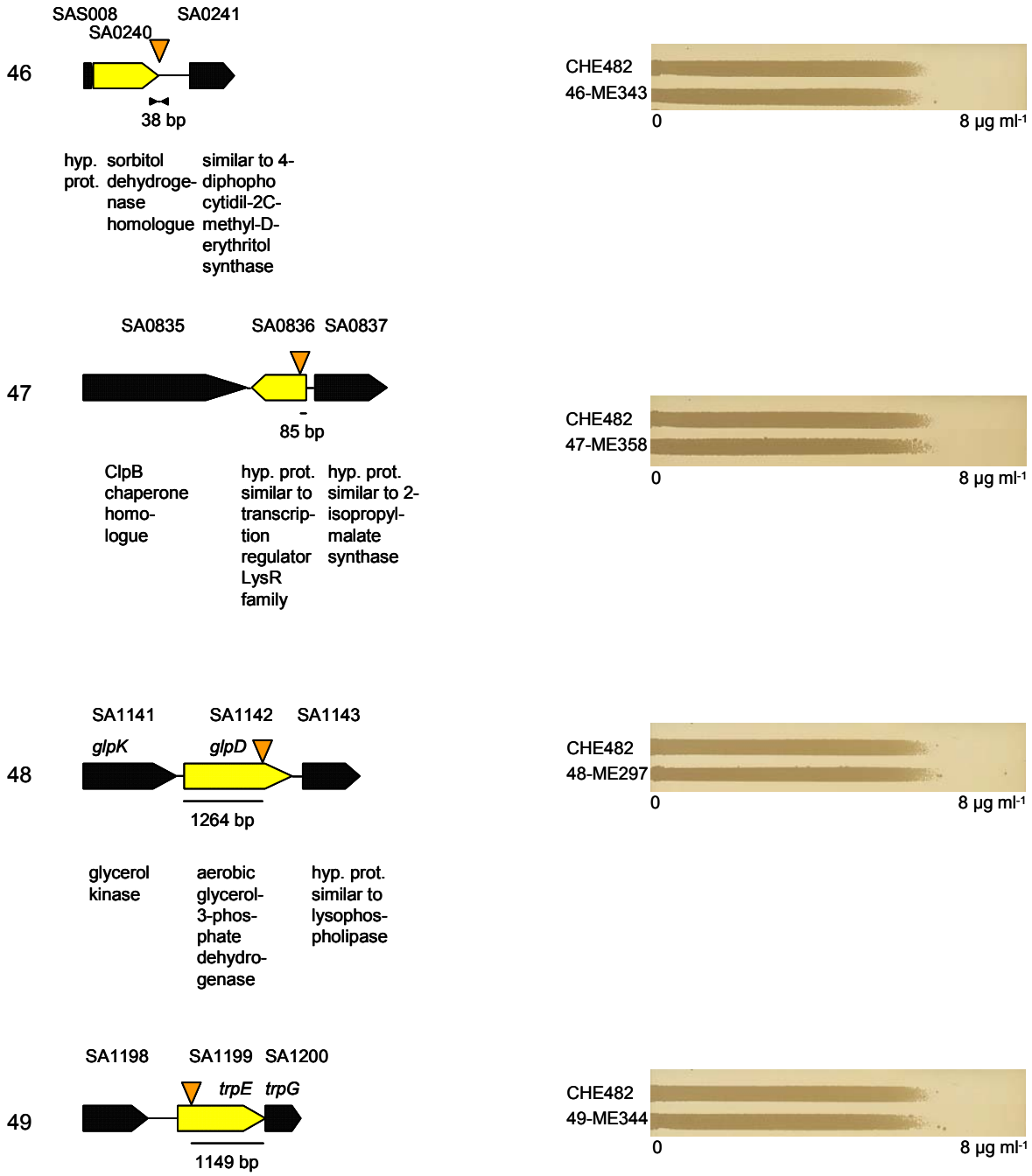

Figure 1 continued.

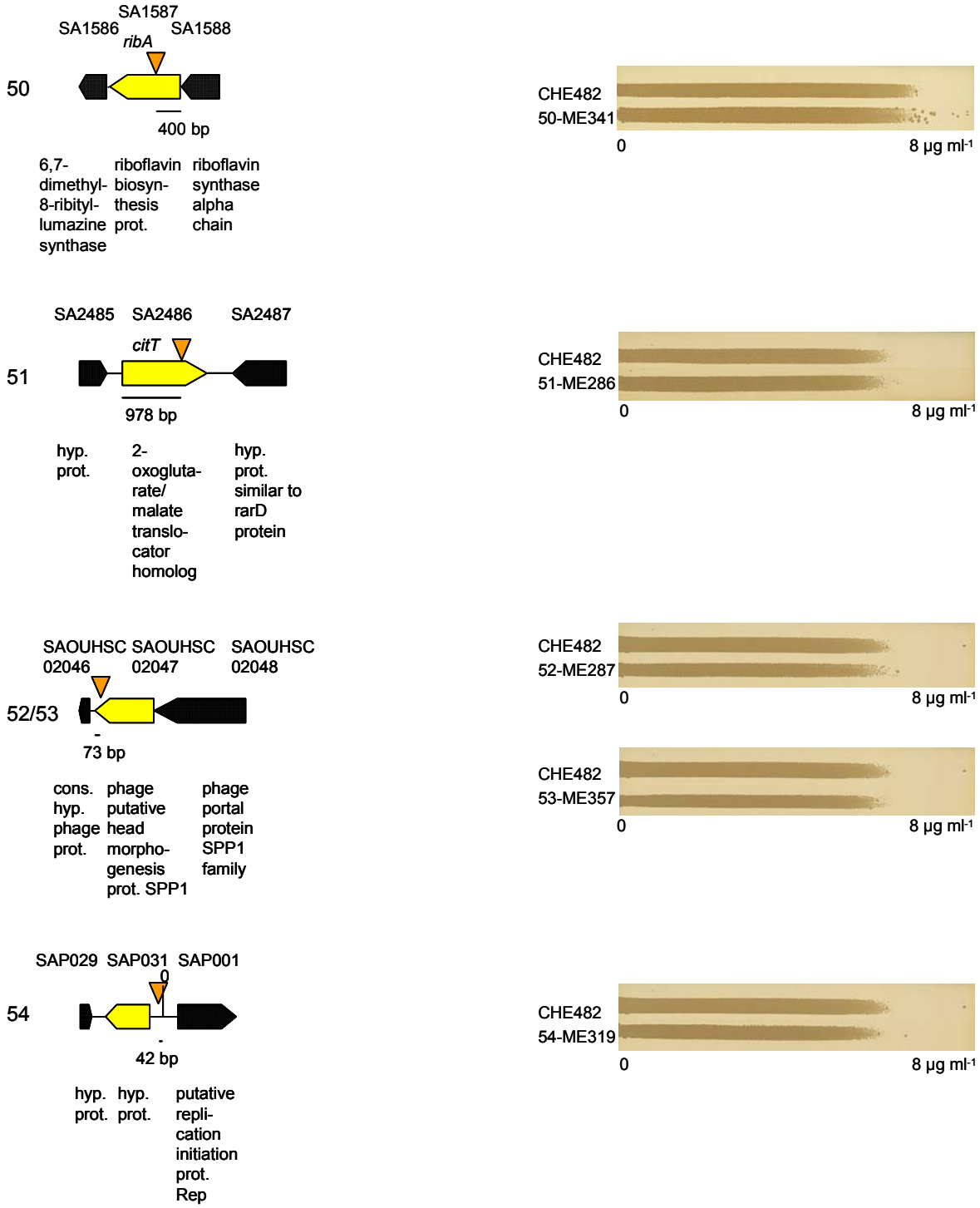

Figure 1.

Schematic illustration of the gene/promoter interrupted by *bursa aurealis* and adjacent genes. The site of integration is indicated by an orange arrow. The distance of the insertion site to next stop or start codon is indicated and displayed by a black line. orf numbers are from strains N315-SA [BA000018], pN315-SAP [AP003139], MRSA252-SAR [BX571856] and NCTC8325-SAOUHSC [CP000253].

Table 3. Number of interrupted orfs expressing altered resistance phenotypes, classified by their cellular role (Tigr database). orf numbers are from N315 (SA) , pN315 (SAP), MRSA252 (SAR).

Nr.	Cellular role category	Orf number
5	Unclassified	SA0370, SA0423, SA0463, SA1564, SA1661
4	Cell envelope	SA0038, SA0462, SA0931, SA1874
3	Transport and binding proteins	SA0809, SA2137, SAR1775
2	DNA metabolism	SA0461, SA1501
2	Energy metabolism	SA1063, SA2279
2	Amino acid biosynthesis	SA0756, SA1563
1	Protein fate	SA1463
1	Regulatory function	SAP011
1	Biosynthesis of cofactors etc.	SA1054
21	total	

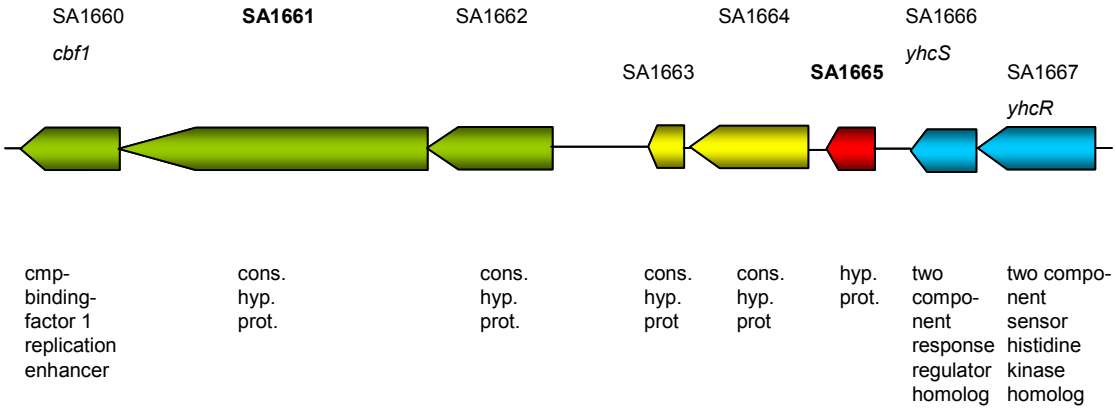

Figure 2.

Organisation of the putative operons covering orfs SA1660 to SA1667 (putative operons were identified using http://www.softberry.com). The operons are coloured green (SA1660-SA1662), yellow (SA1663-SA1664), blue (SA1666-SA1667), and the SA1665 single orf transcript is shown in red. Putative functions are indicated, if known. Genes shown to affect methicillin resistance are in bold type.

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4. Comments – Outlook

The variability in SCC*mec*$_{N1}$ spreading among injection drug users in Zurich, Switzerland suggests that SCC*mec* is a dynamic structure, even within a rather small and seemingly localized MRSA clone. To follow the further spread and evolution of this so called dug clone the following questions need to be addressed: Will new variants of SCC*mec*$_{N1}$ continue to arise? Will the drug clone acquire additional chromosomal resistances over time? Will clones of higher β-lactam resistance emerge and whether or not the elevated resistance will cause a fitness loss? Will drug clones spread outside the drug user community to other environments or regions? Is the ratio drug clone versus other MRSA clones increasing or decreasing over time?

Following the evolutionary process of this clone and its SCC*mec* might be a complex task because several variants have already been detected and as continuous changes in the chromosome and SCC*mec* occur, SCC*mec* heterogeneity is likely to increase.

Evidence that a *mecA* promoter mutation had only a minor effect on β-lactam resistance levels and that unknown chromosomal genes had a higher impact, confirms that the strains genomic background is of considerable importance for β-lactam resistance, not only determining heterogenity/homogenity but also how high or low a strains resistance levels will be.

A novel DNA-binding protein (SA1665) was identified and shown to indirectly modulate β-lactam resistance in *Staphylococcus aureus.* Although it binds to the *mecA* coding DNA it does not influence *mecA* transcription or PBP2a levels. This novel transcription factor might regulate several factors but those relevant in β-lactam resistance need to be identified. Potentially regulated chromosomal factors might be previously discovered *fem/aux* factor/s or a completely new factor/s of which the function will have to be elucidated.

The random transposon insertion library of CHE482, a low level methicillin resistant *S. aureus*, allowed the identification of novel *fem/aux* factors that have an impact on β-lactam resistance, showing that not all genes affecting β-lactam resistance have been identified. To extend this list even further, screenings of strains with different clonal backgrounds and resistance levels would be necessary, as some genes may have very large effects on β-lactam resistance levels in some strains but not others. Identification of the genes responsible for low/high and heterogeneous/homogeneous β-lactam resistance expression will be difficult as several factors most probably contribute to this phenotype, and resistance levels may not just depend on the absence or presence of these genes but also on their expression levels.

5. Appendix

5.1 Acknowledgement

A special thanks goes to Prof. Dr. Brigitte Berger-Bächi for her ideas, motivation, effort and time in supervision and revision of this work.

I would like to thank Prof. Dr. Leo Eberl, my faculty supervisor for his organizational help and Prof. Dr. Jakob Pernthaler who, together with Prof. Dr. Leo Eberl, agreed to be members of my exam committee.

Dr. Nadine McCallum, my supervisor, she helped me to find solutions, always had new ideas how to go on and finally corrected my English style and grammar, so thank you for your support.

Thanks to Prof. Dr. Greg Cook who gave me the opportunity to work in his lab at the University of Otago, where I could learn about continuous cultures.

I would like to thank the whole research group of the BB lab for the great atmosphere, all the discussions, ideas, help and especially for the coffee breaks, which I will miss most.

Everybody who encouraged and cheered me up during harder time periods, I would like to say thank you. And to those people who where always asking, when I will finally finish let me say: “You do not know what you are talking about!”

Dennis for his help with all my not infrequent problems with the computer, filling tips ;o) and for listening to my problem in the lab even if he sometimes had no idea what I was talking about, thank you.

Thank you to my big sister Isabell who still takes care of her little sister!!!

To my Parents I would like to say: „Thank you very much for your encouragement, support and patience during my studies. It has taken a little bit longer than we all thought, but you never doubted that I can make it, you are the best parents ever!“

5.2 Curriculum vitae

Personal data	
Name	ENDER
First Name	Miriam
Date of Birth	25.06.1978
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Education	
2004 - 2008	Doctoral work at the Institute of Medical Microbiology, University Zürich
2004	Graduation at the Institute of Medical Microbiology, University of Zurich
1998 - 2004	Study of Microbiology at the University of Zurich
1994 - 1998	Grammar school at the Gymnasium Heerbrugg, SG, Typus C

Publications resulting from PhD studies

2007	**Ender M**, Berger-Bächi B, McCallum N. Variability in SCC*mec*$_{N1}$ spreading among injection drug users in Zurich, Switzerland. BMC Microbiology: Jul; 7(62)
2007	Lee SM, **Ender M**, Smith JMB, Adhikari R, Berger-Bächi B, Cook GM. Fitness cost of SCC*mec* in methicillin-resistant *S. aureus* by way of continuous culture. Antimicrob. Agents Chemother. Apr; 51(4): 1497-9
2007	Heusser R, **Ender M**, Berger-Bächi B, McCallum N. Mosaic staphylococcal cassette chromosome *mec* (SCC*mec*) containing two recombinase loci and a new *mec* complex, B2. Antimicrob. Agents Chemother. Jan; 51(1):390-3.

2005	Qi W, **Ender M**, O'Brien F, Imhof A, Ruef C, McCallum N, Berger-Bächi B. Molecular epidemiology of methicillin-resistant *Staphylococcus aureus* in Zürich, Switzerland (2003): Prevalence of type IV SCC*mec* and a new SCC*mec* element associated with isolates from intravenous drug users. J. Clin. Microbiol. Oct; 43(10):5164-70.
Accepted	**Ender M**, McCallum N, Berger-Bächi B. Impact of *mecA* promoter mutations on *mecA* expression and β-lactam resistance levels. Int. J. Med. Microbiol.
Accepted	Berger-Bächi B, Senn M, **Ender M**, Seidl K, Hübscher J, Schulthess B, Heusser R, Stutzmann Meier P, McCallum N. Resistance to β-lactam antibiotics. *In* Archer GL (ed.), The staphylococci in human disease, 2nd ed. Blackwell publ.

In Preparation

Ender M, McCallum N, Berger-Bächi B. A novel DNA-binding protein modulating β-lactam resistance in *Staphylococcus aureus*.

Conference presentations

2007	Ender M, McCallum N, Berger-Bächi B. Variability in SCC*mec*$_{N1}$ (type VIb) spreading among injection drug users In Zürich, Switzerland. 66th SSM –SGM. Interlaken. 1st to 2nd March. Poster award, 3rd prize.
2006	Ender M, McCallum N, Berger-Bächi B. Characterisation of the Swiss MRSA clone spreading among intravenous drug users. 65th SSM –SGM. Lausanne. 7th to 8th March.
2005	Ender M, McCallum N, Berger-Bächi B. Characterisation of the Swiss MRSA clone spreading among intravenous drug users. Gordon Research Conference on Staphylococcal Disease. Newport, RI, USA. 21st to 26th August.
2004	Ender M, McCallum N, Berger-Bächi B. Characterisation of the Swiss MRSA clone spreading among intravenous drug users. 3rd SWIMM Meeting. Zürich. 15th to 17th March.

5.3 Additional publications

Diploma thesis: Fitness cost of SCC*mec* and methicillin resistance levels in *Staphylococcus aureus*.

Acquisition of new resistance determinants or mutations can affect bacterial fitness. To determine the impact of SCC*mec* on *S. aureus* a type I SCC*mec* was transformed into a naïve methicillin susceptible strain. The resulting MRSA expressed high homogeneous methicillin resistance but had a reduced growth rate. Subsequent excision of the SCC*mec* restored the growth rate of the original MSSA strain. When the high homogeneously resistant strain was grown in competition with the cured variant it only survived by segregating faster growing heterogeneously resistant variants. Therefore the acquisition of SCC*mec*, conferring high-level methicillin resistance imposes a fitness burden on naïve MSSA. Fitness can be regained in these strains but at the cost of lowered resistance.

Collaboration: Molecular epidemiology of methicillin-resistant *Staphylococcus aureus* in Zurich, Switzerland (2003): Prevalence of type IV SCC*mec* and a new SCC*mec* element associated with isolates from drug users.

Epidemiological analyses were performed on MRSA isolated from the Zurich area in 2003. Characterisation included determination of SCC*mec* type, resistance profile and genetic lineage. Typical hospital acquired SCC*mec* type I/II/III were found in 26 % of isolates, while 41 % contained SCC*mec* type IV. Twenty-six strains carried untypable SCC*mec* elements, of which 22 belonged to a single clone with a CC45/ST45 MLST type, and low level oxacillin resistance. Other distinguishing features included a novel SCC*mec* element with a *ccrAB4*-like recombinase, and trimethoprim resistance conferred by *dfrA* of Tn*4003*. This clone was found among injection drug users in the area of Zurich and therefore referred as a “drug clone”. Different variants of this clone have already been found, some containing either an additional sulfomethoxazole or ciprofloxacin resistance. MRCNS had a completely different pattern of SCC*mec* types and no drug clone SCC*mec* was identified, indicating that no considerable interspecies transfer occurred between MRSA and MRCNS populations.

Contribution: Implementation of the SCC*mec* typing and molecular characterisation of the new SCC*mec* element of the “drug clone”.

Collaboration: Fitness cost of staphylococcal cassette chromosome *mec* in methicillin-resistant *Staphylococcus aureus* by way of continuous culture.
Integration of mobile genetic elements can often result in decreased fitness, unless compensatory mutations occur. Isogenic transformants containing either SCC*mec* type I or IV were investigated and their effect on fitness determined by the use of continuous cultures. Comparison of glucose consumption, ATP demand per gram of cells and cell yield showed a decreased growth yield after the acquisition of type I SCC*mec* whereas type IV had no adverse effect on fitness.
Contribution: Implementation of the media, run of the continuous cultures, measurements of dry weight, glucose consumption and ATP production - together with Sui Mae Lee. A third measurement was performed by Sui Mae Lee.

Supervision: Mosaic staphylococcal cassette chromosome *mec* containing two recombinase loci and a new *mec* complex, B2.
In the epidemiological study at the Institute of Medical Microbiology, University Zurich in 2003, a new type of staphylococcal cassette chromosome *mec* (SCC*mec*) element was detected, which seemed to harbour both *ccrAB2* and *ccrC*. Cosmid cloning and sequencing of this element identified a new mec complex (B2) interrupted by Tn*4001*, conferring aminoglycoside resistance, and confirmed the presence of both *ccrAB2/ccrC* loci. Sequence comparisons revealed that this element had a mosaic structure and that several recombination events were likely to have occurred during its assembly.

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Fitness Cost of SCC*mec* and Methicillin Resistance Levels in *Staphylococcus aureus*

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Transformation of a type I SCC*mec* element into *Staphylococcus aureus* yielded highly oxacillin-resistant transformants with a reduced growth rate. Faster-growing variants could again be selected at the cost of reduced resistance levels, demonstrating an inverse correlation between oxacillin resistance levels and growth rate.

The development of antibiotic resistance in bacteria through either the acquisition of resistance elements or mutation often occurs at the cost of reduced fitness and may result in a decreased bacterial growth rate (1). Evolution in the natural or the clinical environment usually selects for fitter variants, which compensate for the cost of resistance through the development of secondary mutations or the loss of the resistance (10).

Methicillin resistance in methicillin-resistant *Staphylococcus aureus* (MRSA) is mediated by the acquisition of the SCC*mec* element, which integrates in a site-specific manner into the staphylococcal genome (7). Besides the *mecA* gene, which codes for a penicillin-binding protein (PBP) with a low affinity to β-lactams, it harbors a variable set of genes unrelated to methicillin resistance and serves as an integration site for various other resistance determinants, transposons, and plasmids. Establishment of the SCC*mec* element in staphylococci theoretically encounters two main obstacles: one is the cost of maintenance of a relatively large additional element, of which only the *mecA* gene is essential for resistance; the second is the accommodation of the new PBP 2a into the staphylococcal cell wall synthesis complex. Initially, when PBP 2a enters a naïve *S. aureus* strain and in the absence of β-lactam pressure, PBP 2a is not beneficial and the cells select against its production (8). The ability of *S. aureus* to accommodate SCC*mec* and/or to functionally integrate PBP 2a differs from strain to strain, resulting in a wide range of resistance levels (3). Irrespective of their original oxacillin resistance levels, MRSA strains are resistant to all β-lactam antibiotics due to their ability to segregate highly resistant variants. Multiple different mutations may lead to high levels of resistance (for reviews, see references 13 and 14), but few of them have been identified (4, 9).

Our goal was to measure the in vitro cost of SCC*mec* on fitness by monitoring changes in growth rates. We transformed naïve, susceptible strain BB255, a derivative of the widely used strain NCTC8325 (6), by the $CaCl_2$ method (2) with DNA originating from an MRSA strain containing a type I SCC*mec* element. The transformants were selected on plates containing 4 μg of cefoxitin per ml. Resistance tests were performed according to the recommendations of the National Committee for Clinical Laboratory Standards (11). The transformants were highly oxacillin resistant (MICs, 512 μg/ml). The stability of the high-level-methicillin-resistance phenotype of representative strain RA120 was tested by dilution of an RA120 culture and subsequent regeneration from single cells in nonselective medium, which demonstrated that the high-level-resistance phenotype was not an induction phenomenon resulting from selection on cefoxitin.

Interestingly, the transformants grew much slower than the susceptible parent, with a generation time of 40 ± 0.1 min compared to a generation time of 29 ± 0.1 min for strain BB255. Strain RA120 was cured of SCC*mec* by transient overexpression of *ccrAB* from plasmid pSR3-1, which induces precise, site-specific excision of SCC*mec*, as described by Ito et al. (5). The resulting susceptible strain, strain ME23, regained the doubling time and the chromosomal SmaI pulsed-field gel electrophoresis (PFGE) pattern of wild-type strain BB255 (Fig. 1), demonstrating the excision of SCC*mec*. This decrease in the growth rate after the introduction of SCC*mec* and the subsequent increase in the growth rate upon curing confirmed that SCC*mec* and/or the resulting high oxacillin resistance level was the cause of the decreased growth rate and, thus, the loss of fitness in vitro.

Mixed growth competition assays were performed between strain RA120 and susceptible strain ME23 by inoculating 10^4 CFU of each strain into 10 ml of Luria-Bertani broth in the absence of antibiotic pressure. Where indicated, the ratio of RA120 to its competitor was raised to 100:1 by increasing the RA120 inoculum to 10^6 CFU. Every 24 h the mixed culture was diluted by a factor of 10^4 with fresh broth, and the number of CFU of the susceptible strain per milliliter versus that of the resistant strain was determined by plating aliquots on nonselective plates and on plates containing 1 μg of oxacillin per ml and calculating the difference in the number of CFU. At an initial ratio of RA120 to ME23 of 1:1, the RA120 population was lost at a rate of 2 $\log_{10}$ CFU per day (Fig. 2a). Increasing the ratio of RA120 to ME23 to 100:1 allowed the faster-growing variants, represented by strain ME51, with a doubling time of 29 ± 0.1 min, to emerge (Fig. 2b) and to compete

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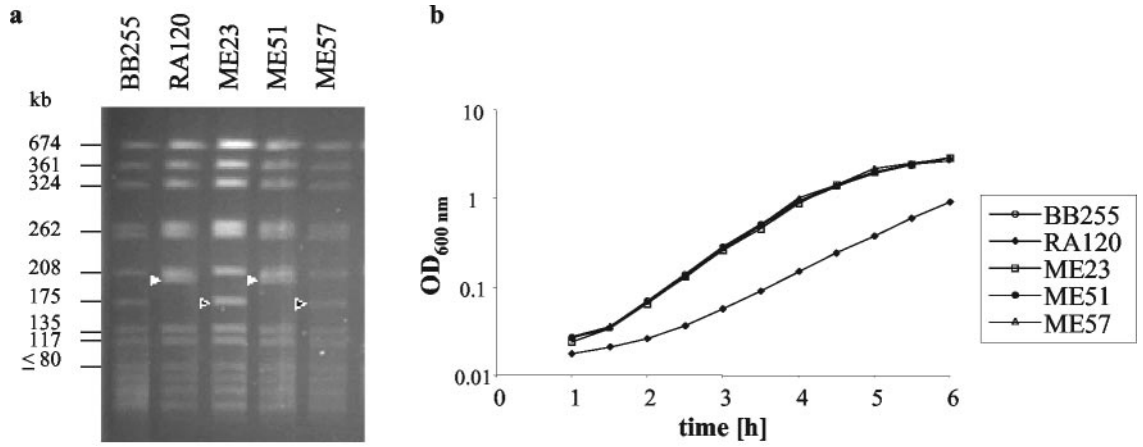

FIG. 1. SmaI restriction patterns of the strains used and their corresponding growth curves. The SmaI fragments carrying SCC*mec* are indicated by filled triangles, and the corresponding fragments of the cured strain are indicated by open triangles. BB255, naïve, susceptible recipient; RA120, BB255 transformant containing SCC*mec*; ME23, RA120 cured of SCC*mec*; ME51, rapidly growing strain derived from RA120; ME57, ME51 cured of SCC*mec*.

successfully with ME23 (Fig. 2d). The doubling time of this faster-growing variant, ME51, remained constant when it was retested 10 days later, and the strain maintained the same restriction pattern as RA120 (Fig. 1). Subculturing of RA120 alone under the same conditions did not yield faster-growing variants after the same number of days, indicating that faster-growing mutants were selected only in the presence of a competitor.

When ME51 was cured of SCC*mec*, the resulting strain, strain ME57, was indistinguishable by its growth rate and PFGE pattern (Fig. 1) from BB255 and ME23. The results of competition experiments with RA120 and ME57 were identi-

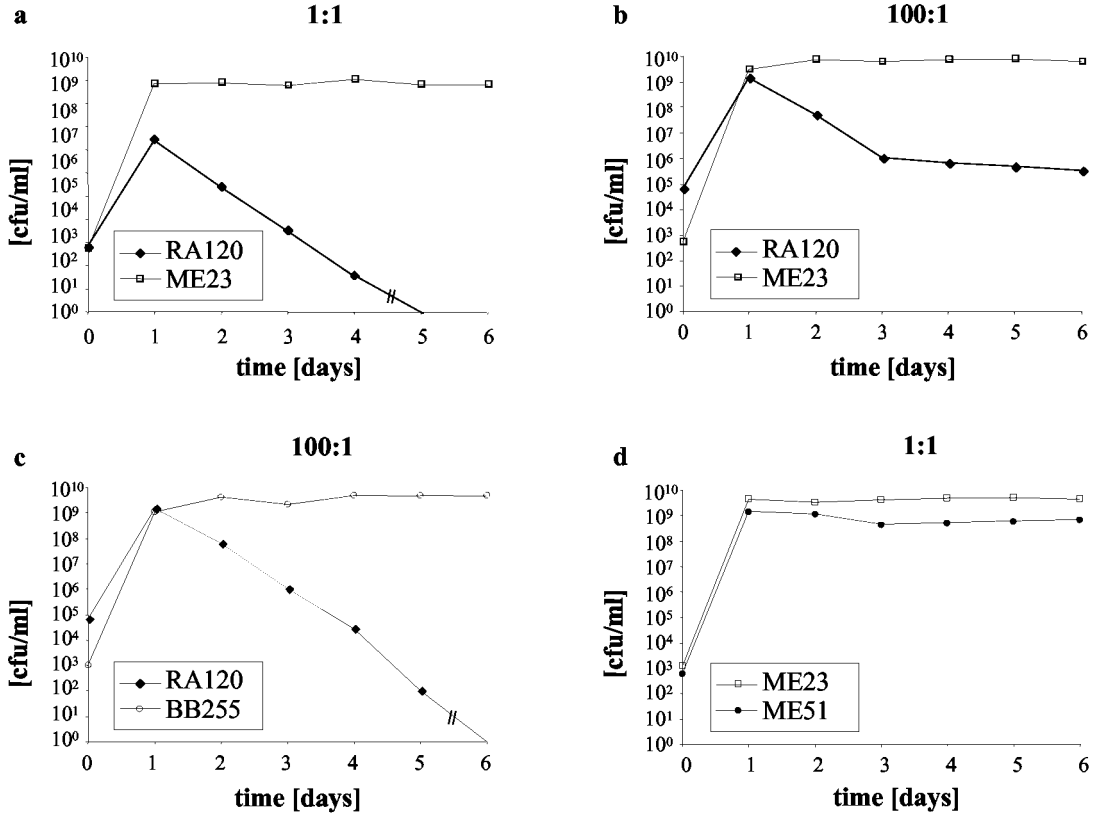

FIG. 2. Growth competition. Survival of oxacillin-resistant strains versus that of oxacillin-susceptible strains in mixed culture after daily subculturing. (a) Strain RA120 with its cured derivative ME23 inoculated at a ratio of 1:1; (b) strain RA120 with ME23 inoculated at a ratio 100:1; (c) strain RA120 with BB255 inoculated at a ratio of 100:1; (d) strain ME51 with ME23 inoculated at a ratio 1:1.

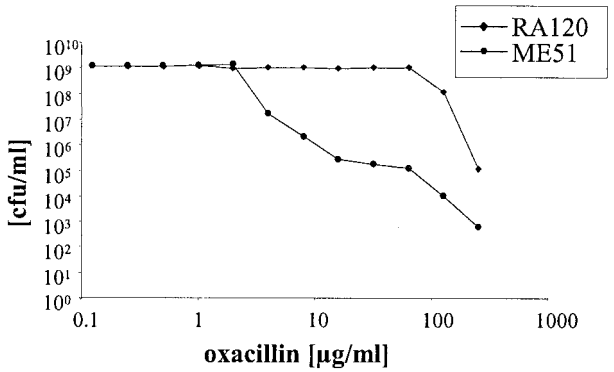

FIG. 3. Population analysis profiles. The numbers of colonies that formed after 48 h in the presence of different concentrations of oxacillin after overnight cultures of strain RA120 or ME51 were plated on increasing concentrations of oxacillin are indicated.

cal to those of competition experiments with RA120 and ME23 (data not shown). However, when RA120 was grown in competition with naïve strain BB255 (Fig. 2c), it was more rapidly eliminated than it was in competition experiments with ME23 or ME57. This suggests that ME23 and ME57 were not as competitive against RA120 as BB255 was; this is possibly due to the acquisition of a chromosomal mutation, as has previously been postulated to occur in highly resistant MRSA strains (14). However, the introduction of an accidental mutation upon transformation cannot be ruled out.

An interesting observation was that strain ME51, as well as 24 of 24 other fast-growing oxacillin-resistant single colonies analyzed from the competition of strains RA120 and ME23, all had reduced levels of oxacillin resistance compared to that of RA120. However, at this stage we do not know whether the fast-growing population comprised a single clone or whether several events resulted in faster growth. The population analysis profile revealed that RA120 had changed from a homogeneously, highly resistant MRSA strain into a heterogeneously resistant MRSA strain (Fig. 3) for which the oxacillin MIC was 64 μg/ml. Highly resistant subclones of ME51, isolated and purified from plates containing 128 or 256 mg of oxacillin per ml, again showed a reduced growth rate, with a doubling time of 39.5 ± 2.7 min. Analogous transformations into BB255 were done with other SCC*mec* type I or type IV elements (data not shown), and analysis of the generation time confirmed a correlation between oxacillin resistance levels and growth rate.

The cost of SCC*mec* may be compensated for in nature. This is reflected by the rapid rate of growth of community-acquired MRSA strains upon which pressures other than antibiotics may act and which, interestingly, have generally been found to exhibit lower oxacillin resistance levels (12). We cannot exclude the possibility that the experimentally demonstrated interrelationship between oxacillin resistance levels and growth rate may also be compensated for in clinical isolates. Oxacillin resistance levels appear to have a higher impact on the growth rate than the addition of the extra DNA comprising the SCC*mec* element.

We thank T. Ito for plasmid pSR3-1.

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Molecular Epidemiology of Methicillin-Resistant *Staphylococcus aureus* in Zürich, Switzerland (2003): Prevalence of Type IV SCC*mec* and a New SCC*mec* Element Associated with Isolates from Intravenous Drug Users

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The majority of methicillin-resistant *Staphylococcus aureus* (MRSA) isolates, recovered in 2003 at the Department of Medical Microbiology in Zürich, Switzerland, belonged to major clones that are circulating worldwide. Staphylococcal cassette chromosome *mec* type IV (SCC*mec*-IV), harbored by half of the isolates, was found in sequence type 217 (ST217), which is an allelic variant of epidemic MRSA-15 (designated EMRSA-15), in a new local ST617 descending from clonal complex CC8 and in low-level oxacillin-resistant strains of multiple genetic lineages characteristic of community-onset MRSA. SCC*mec*-I, SCC*mec*-II, and SCC*mec*-III were in the minority, and four MRSA isolates had complex, rearranged SCC*mec* elements. A novel SCC*mec*-N1 of approximately 30 kb, associated with a *dfrA* gene and a *ccr4*-related recombinase complex, was identified in a large number of low-level oxacillin-resistant isolates, which descended from the successful clonal complex CC45 and are spreading among intraveneous drug users. In contrast, the SCC*mec* types of oxacillin-resistant coagulase-negative staphylococci (MRCNS) were of completely different composition. SCC*mec* type I (SCC*mec*-I) and SCC*mec*-II were more frequent than in the MRSA, while fewer contained SCC*mec*-IV. The other MRCNS displayed 11 different, complex patterns, suggesting frequent recombination between different SCC*mec* elements. With one *ccr*-negative exception, these strains amplified between one and three different *ccr* products, indicating either new varied complexes or multiple *ccr* loci. This suggests the presence of novel SCC*mec* types in MRCNS and no extensive interspecies SCC*mec* transfer between MRSA and MRCNS.

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major cause of hospital-acquired infections and has also recently established itself as a significant community-acquired pathogen (7, 9). Community-onset MRSA (cMRSA) differs from nosocomial MRSA in that it does not generally belong to the major clonal groups of epidemic MRSA, is susceptible to most non-β-lactam antibiotics, contains the type IV SCC*mec* (for staphylococcal cassette chromosome *mec*, the mobile genetic element encoding methicillin resistance), and frequently carries genes responsible for the production of Panton-Valentine leukocidin (PVL) (13, 22, 26). In contrast, nosocomial MRSAs are generally multidrug resistant and contain SCC*mec* types I, II, or III.

Besides the five major allelic types of SCC*mec* elements, epidemiological studies paired with molecular characterizations suggest the existence of additional, different SCC*mec*s (16, 28, 36). The origin of the SCC*mec* element is still unclear, with the closest *mecA* homolog found in *Staphylococcus sciuri* (43). Methicillin-resistant coagulase-negative staphylococci, which are more frequently carriers of SCC*mec* than *S. aureus*, are postulated to be the reservoir for the transfer of methicillin resistance to *S. aureus* (2). SCC*mec* has the attributes of a mobile element, such as the *ccr* genes, encoding recombinases that were shown in vitro to be responsible for the precise excision and integration of SCC*mec* into the chromosome. However, the type 1 and type 3 *ccrA* and *ccrB* genes are dysfunctional. This, plus the larger size of nosocomial SCC*mec* types I to III, may have been the reason that they have spread only into a restricted number of genetic lineages, whereas the smaller type IV SCC*mec* seems to be more mobile and to be associated with more diverse strain lineages (29, 32).

The ability of MRSAs to segregate a highly resistant subpopulation in the presence of β-lactams makes them resistant to virtually all β-lactams and their derivatives. The level of oxacillin resistance reached is strain specific and can vary over a 1,000-fold concentration range, depending upon the genetic background of the strain. On the one hand, it depends upon the ability of MRSA to rapidly induce the synthesis of penicillin-binding protein 2a (PBP2a), which is indispensable for resistance; on the other hand, it is the genetic background of the strain into which the SCC*mec* element has entered which determines the final resistance level (for a review, see reference

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5). Some clinical MRSA isolates have such a low level of resistance to oxacillin that they are difficult to identify phenotypically. Interestingly, an inverse relationship between resistance levels and growth rate has been observed (10).

The recent appearance of vancomycin-intermediate-resistant MRSA (VISA), which is due to alterations in gene expression caused by gene induction and/or the accumulation of multiple mutations which finally lead to intermediate levels of glycopeptide resistance (17), is a further challenge for the diagnostic laboratory. VISAs may go undetected by conventional resistance tests, since they produce only a small number of cells within a culture that express resistance. VISAs are still regarded as a rare cause of clinically relevant infections (35), but the evolution of their prevalence needs to be monitored.

The aim of this study was to examine the SCC*mec* types, the associated resistances, and clonal composition of MRSA isolates in the Zürich area and to search for a correlation between low-level oxacillin-resistant strains, which are increasingly being isolated, and the genetic background and/or SCC*mec* type. A comparison of the SCC*mec* elements occurring in methicillin-resistant coagulase-negative staphylococci (MRCNS) with those in MRSA should show if the distribution of SCC*mec* in MRSA is a reflection of that in MRCNS.

MATERIALS AND METHODS

Bacterial strains. Ninety independent MRSA isolates and 88 randomly selected coagulase-negative, methicillin-resistant staphylococcal isolates (MRCNS) sampled between November 2002 and January 2004 at the Department of Medical Microbiology of the University of Zürich, Zürich, Switzerland, which serves the university hospitals of Zürich, were analyzed. All strains were unique isolates from different patients. Identification of *S. aureus* and of the coagulase-negative staphylococci was by standard methods: colony morphology, Gram staining, catalase test, and confirmation with Staphaurex (Murex Diagnostics) and API Staph (bioMérieux), where required. Oxacillin resistance was confirmed by *mecA* PCR and with the MRSA screen from Denka Seiken (Japan) using β-lactam-induced bacteria growing around the amoxicillin-clavulanic acid inhibition zone for the detection of PBP2a (PBP2′) by latex agglutination (44). The strains were stored in skim milk at −80°C. Reference strains were *S. aureus* NCTC10442 for SCC*mec* type I, *S. aureus* N315 for SCC*mec* type II, *S. aureus* 85/3907 for SCC*mec* type III (19), *S. aureus* WSPP for SCC*mec* type IV (1, 25), and *S. aureus* WBG8404 for SCC*mec* type V (20). The Panton-Valentine leukocidin-positive WSPP strain and Swiss strain 497 (22) were used as references for *lukS-PV*. Mu3, a hetero-VISA (hVISA) strain, and susceptible strain N315 (18) were used as comparisons for glycopeptide intermediate resistance in population analysis profiles. The quality control strains for antibiotic resistance testing were *S. aureus* ATCC 29212 and *Enterococcus faecalis* ATCC 29213. Curing of SCC*mec* from clinical isolates was done by *ccr* overexpression, as described by Ito et al. (19).

Susceptibility testing. Oxacillin, cefoxitin, tetracycline, gentamicin, ciprofloxacin, erythromycin, and clindamycin susceptibility were determined by disk diffusion according to CLSI (formerly NCCLS) (7a) on Mueller-Hinton agar (Difco). Inducible macrolide-lincosamide-streptogramin B (MLS_B) resistance was identified as a D-shaped inhibition zone by the clindamycin-erythromycin double-disk test (21). Oxacillin and linezolid MICs were determined by Etest (AB Biodisk, Solna, Sweden) on Mueller-Hinton agar (Difco) with an inoculum of 0.5 McFarland standard as recommended. Rifampin, cotrimoxazole, and fosomycin MICs were determined by agar dilution according to the CLSI (7a). Vancomycin and teicoplanin resistance were determined by macro Etest on brain heart infusion plates (BBL), using a inoculum consisting of a 2 McFarland standard as recommended by the manufacturer (AB Biodisk, Solna, Sweden). Production of a penicillinase was shown qualitatively by nitrocefin hydrolysis from β-lactam-induced cells growing around an amoxicillin-clavulanate disk. The hVISA phenotype was confirmed by population analysis profile, by plating aliquots of an overnight culture on brain heart infusion agar containing increasing concentrations of either vancomycin or teicoplanin and reading the CFU after a 48-h incubation, using strain Mu3 as a control (15). The growth temperature was 35°C.

Molecular typing. (i) PFGE. The MRSAs were genotyped by pulsed-field gel electrophoresis (PFGE) of SmaI-digested chromosomal DNA, following the protocol of Wada et al. (39). The banding patterns were analyzed visually, by scanning with a Fluor-S MultiImager, and by digital analysis with a Multi-analys/PC (Bio-Rad) (28).

(ii) MLST. Multilocus sequence typing (MLST) was performed with selected isolates as specified by Enright et al. (11). The sequences obtained were compared with the sequences at the MLST website (http://www.mlst.net/) to assign a sequence type (ST).

(iii) *SCCmec* typing. Determination of SCC*mec* types I to IV was done by multiplex PCR (30). Untypable strains were further analyzed by *ccr* typing using a PCR screen with primers to identify *ccr* types 1, 2, and 3 (19); the SCC*mec* type V *ccr* complex *ccrC* gene (20); and primers C1 and C2 (23) to detect the *ccr4* locus of the pediatric clone (31).

(iv) PVL. The primers lukSF (5′-ACAGAAGATACAAGTAGCGA-3′) and lukSR (5′-TAATTCATTGTCTGGCACAA-3′) were used to detect the presence of the *lukS-PV* gene, which is specific for Panton-Valentine leukocidin, by PCR.

(v) Colocalization of *dfrA-mecA*. Sequential Southern hybridizations (4) of SmaI-digested chromosomal DNA separated by PFGE with a *mecA* (30) or *dfrA* probe, amplified with the primer pair dfrAF-Tn*4003* (5′-AATAGACGTAACG TCGTACT-3′) and dfrAR-Tn*4003* (5′-AAGAATGTATGCGGTATAGT-3′), showed whether *dfrA* mapped in the same SmaI band as *mecA*.

RESULTS AND DISCUSSION

Oxacillin resistance and β-lactamase production. The University Hospital of Zürich, a 920-bed hospital, had an incidence of 1.1 cases of MRSA per 1,000 admissions in 2003. Ninety independent isolates were collected from November 2002 through January 2004 and characterized to determine their resistance profile, clonal distribution, PFGE pattern, and SCC*mec* types (Table 1). For comparison, 88 MRCNS clinical isolates were sampled randomly during the same time span to see if there was any correlation between the SCC*mec* types of MRCNS and MRSA. Methicillin resistance was confirmed in all strains by *mecA* PCR. The frequency of nonmultiresistant MRSA with low-level oxacillin resistance was rather high in this collection. Thirty percent of all MRSA strains had an oxacillin MIC below the breakpoint of 4 mg/ml (Fig. 1). In disk diffusion tests, the 30-mg cefoxitin disk was found to be superior and easier to interpret than the oxacillin disk and correctly identified all low-level resistant MRSA, with only one strain displaying intermediate resistance. The PBP2a agglutination by the MRSA latex-screening test using induced bacteria was as reliable as the *mecA* PCR, even for the phenotypically oxacillin-susceptible MRSA.

While MRSAs displayed oxacillin MICs over the entire range measured, the MRCNS formed two distinct clusters, consisting of 39 strains with oxacillin MICs of ≤32 mg/ml and 49 isolates with oxacillin MICs of ≥256 mg/ml. One MRCNS had an oxacillin MIC below the breakpoint for coagulase negative staphylococci of 0.5 mg/liter. Interestingly, penicillinase production was more frequent in MRSAs (90%) than in MRCNS (74%).

PFGE and *SCCmec* typing of MRSAs. PFGE indicated that there were four epidemiologically dominant genetic lineages and a number of sporadic isolates of MRSAs isolated from the University Hospital of Zürich (Fig. 2). The genetic backgrounds of representatives of each of the larger groups were investigated by MLST and determined to be ST217-MRSA-IV, a single-locus variant (SLV) of the pandemic epidemic United Kingdom strain epidemic MRSA-15 (designated EMRSA-15); ST225-MRSA-II, an SLV of the Japanese/America clone;

TABLE 1. Characteristics of the MRSAs grouped according to SCC*mec* type

No. of strains	No. of different dendrogram profiles	SCC*mec* type	No. of strains containing[a]				MIC_{50}/MIC_{90} Oxa[b]	No. of strains in each group resistant to:[c]							
			dfrA-mecA	*dfrA*	*pvl*	*blaZ*		SXT	Tet	Cm	Gen	Cip	Ero[d]	Cli[d]	Rif
3	3	I					>256/>256				3	3	3	3	3
9	4	II				8	4/>256				1	9	9	6	
11	11	III				11	>256/>256	6	9	1	8	7	7	2	1
41	13	IV		2	5	36	4/128		3		1	23	17	7	2
22	3	N1	22			22	1.5/4	11				11			
1	1	N2				1	>256				1				1
1	1	N3					0.5					1			
2	1	N4				2	>256	1	2		2	2	2	2	2

[a] *dfrA-mecA*, *dfrA* integrated into SCC*mec*; *dfrA,* dihydrofolate reductase gene not associated with SCC*mec*; *pvl,* Panton-Valentine leukocidin; *blaZ*, penicillinase.
[b] Oxa, oxacillin.
[c] SXT, cotrimoxazole; Tet, tetracycline, Cm, chloramphenicol; Gen, gentamicin; Cip, ciprofloxacin; Ero, erythromycin; Cli, clindamycin; Rif, rifampicin.
[d] All clindamycin-resistant strains had constitutive erythromycin resistance, all erythromycin-resistant but clindamycin-susceptible strains had an inducible erythromycin resistance.

ST613-MRSA-IV, a new ST so far reported only in Zürich; and ST45-MRSA-N1, the drug clone, so far predomantly found in isolates from intraveneous drug users and their contacts in Zürich. The ST217 and ST255 isolates have the characteristics of nosocomial MRSAs, while ST613 and ST45 have the characteristics of cMRSA (Table 1). The predominance of SLVs of pandemic isolates, the new ST, and the unique drug clone suggest that these MRSAs have evolved locally and are disseminating in the geographical region.

The classic nosocomial SCC*mec* types I, II, and III formed a minority in this MRSA strain collection with only 3, 9, and 12 isolates, respectively, representing 26% of all isolates. Two MRSAs produced a composite pattern consisting of a partial SCC*mec* type III lacking region C but with region D, otherwise found in types I, II, or IV SCC*mec* elements (Table 2). These two strains contained the *ccr* type 3 recombinase allele and clustered in the PFGE-based dendrogram within the SCC*mec* type III clusters (Fig. 2). They may therefore represent MRSAs with a new, composite SCC*mec* element, listed here as SCC*mec* type N4. Forty-one MRSAs carried SCC*mec* type IV and could be divided according to their PFGE pattern into three major groups (Fig. 2). The predominant clone, consisting of 18 strains with generally high levels of oxacillin and ciprofloxacin resistance, belonged to ST217 of CC22, differing from the ancestral clone EMRSA-15, a strain epidemic in the United Kingdom (12), at the *tpi* locus. The second major group of 10 strains belonged to a new sequence type, ST613, so far only found in Zürich and originating from clonal complex CC8. The remaining type IV strains formed a heterogeneous group with various PFGE patterns, which is common for the small community-onset-type IV SCC*mec* elements with enhanced mobility. PVL, reported to be associated with cMRSAs, was not frequent in this MRSA collection; the *lukS-PV* gene was present only in five SCC*mec* type IV strains. Interestingly, one of the PVL-producing strains was found during an outbreak in a dermatological ward.

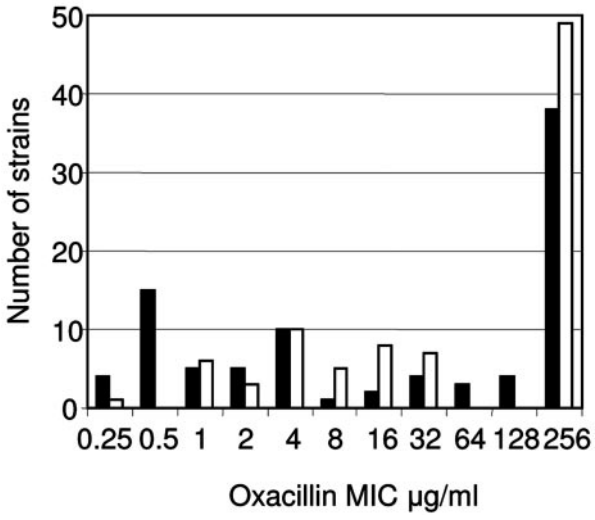

FIG. 1. Distribution of oxacillin MICs. Black bars, MRSA (90 strains); white bars, MRCNS (89 strains).

Characterization of the "drug clone." Twenty-four MRSAs amplified only the *mecA* band with the SCC*mec* multiplex PCR. All isolates, except for three, carried none of the *ccrAB* alleles types 1, 2, or 3 or the *ccrC* gene, but with the primers specific for the *ccr4* complex they yielded a band very closely related to that of the pediatric clone reported by Oliveira et al. (31; M. Ender, unpublished results). Curing the SCC*mec* determinant from a representative of these strains showed, according to the SmaI PFGE banding pattern, that the element had a size of approximately 30 kb. Interestingly, the SmaI band which carried the *mecA* gene also harbored a copy of the Tn*4003*-associated *dfrA* gene, which was lost upon SCC*mec* curing. PCR mapping and sequencing showed that the *dfrA* gene was integrated into the SCC*mec* determinant (Ender, unpublished). The association of *dfrA* with the SCC*mec*, the lack of any characteristic bands of type I to IV SCC*mec*s other than *mecA* by multiplex PCR, and the presence of a *ccr4*-like *ccr* complex suggested that this was a new SCC*mec* element, termed here SCC*mec* type N1. All of these strains belonged to an MRSA clone, which is spreading among isolates from injection drug users (14) and which is referred to as the "drug clone." It is characterized phenotypically by a very low level of oxacillin resistance, the presence of a penicillinase, and a high maximal growth rate of 1.8 h^{-1} in LB broth. All drug clones were trimethoprim resistant and generally either sulfomethoxazole or ciprofloxacin resistant. The drug clone appeared in 1994 and peaked in 2001 (Fig. 3), but it still represents a

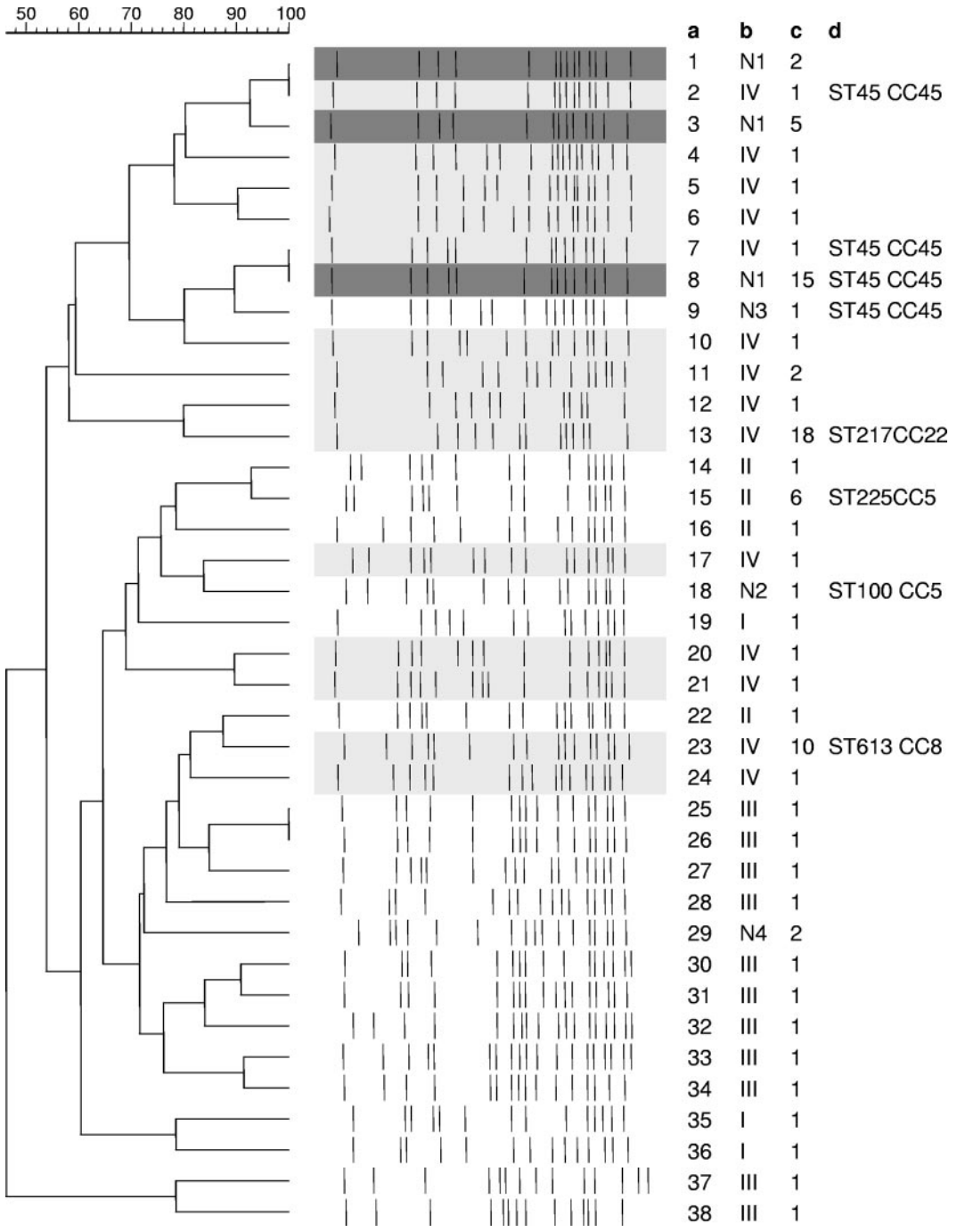

FIG. 2. Dendrogram. a, number of PFGE pattern; b, SCC*mec* type; c, number of isolates of the same PFGE profile and SCC*mec* type; d; sequence type and clonal complex. Light gray shading, SCC*mec*-IV; dark gray shading, SCC*mec*-N1 (drug clone).

substantial part of the MRSA isolates in the Zürich area. It has also spread in a few cases to isolates from non-drug users, probably as a result of nosocomial transmission. MLST showed that the genetic background of the drug clone belongs to allelic profile ST45. The drug clone is thus similar to the epidemic Berlin MRSA clone, with low-level resistance to oxacillin (42), except for carrying the novel SCC*mec* type N1 element.

Two isolates, that like the drug clone only amplified the *mecA* band in the multipex PCR, had novel SCC*mec*s, N2 and N3, that were otherwise unrelated to N1 of the drug clone. They both lacked the *dfrA* gene; moreover, the strain containing SCC*mec* type N2 amplified products specific for the *ccr2* and the *ccr5* complex, whereas the strain containing SCC*mec* type N3 amplified a *ccr2* complex. The strain containing SCC*mec* type N2 was highly oxacillin resistant and also gentamicin and rifampin resistant, very unlike the drug clone. The

TABLE 2. SCC*mec* types and *ccr* complexes identified in the MRSA collection

Amplified region	SCC*mec* type				New SCC*mec* type			
	I	II	III	IV	N1 drug clone	N2	N3	N4
Locus, gene[a]								
A, *pls*	+							
F, Tn544-*orfX*			+					+
G, IS*431*-pUB110		+						
D *dcs*	+	+		+				+
B, *kdp*		+						
E, pl258-Tn*544*			+					+
C, *mecR1*		+	+					
mecA	+	+	+	+	+	+	+	+
ccr complex								
ccr1	+							
ccr2		+		+		+	+	
ccr3			+					+
ccr4					+			
ccr5						+		
No. of isolates	3	9	11	41	22	1	1	2

[a] Loci F, G, and E are located between the orfs/elements indicated, as described by Oliveira and de Lencastre (30).

strain containing SCC*mec* type N3, a ciprofloxacin-resistant strain of low-level oxacillin resistance, was one of a cluster of ST45 isolates that harbored at least three different SCC*mec*s (types N1, N3, and IV) (Fig. 2), indicating that there have been three genetic events during which ST45 isolates found in this region have acquired methicillin resistance.

***SCCmec* typing of MRCNS.** The distribution of SCC*mec* types in the MRCNS showed a completely different pattern. Among the MRCNS, we identified 14 strains with SCC*mec* type I, 12 strains with type II, none with type III, 8 strains with type IV, and a large number of untypable variants, which produced 11 new patterns by multiplex SCC*mec* and *ccr* typing. None of the MRCNS strains was a carrier of the new SCC*mec* type N1 found in the drug clone, since isolates amplifying *mecA* alone carry no *ccr4* complex and no *dfrA* gene, suggesting that MRCNS were unlikely to have been the donors of the SCC*mec*

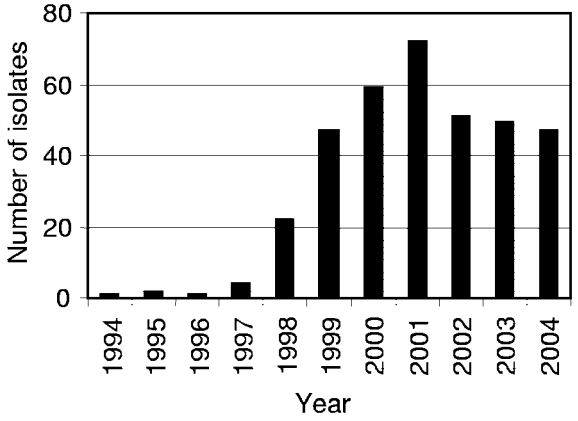

FIG. 3. Number of drug clones isolated at the university hospitals of Zürich from 1994 through 2004.

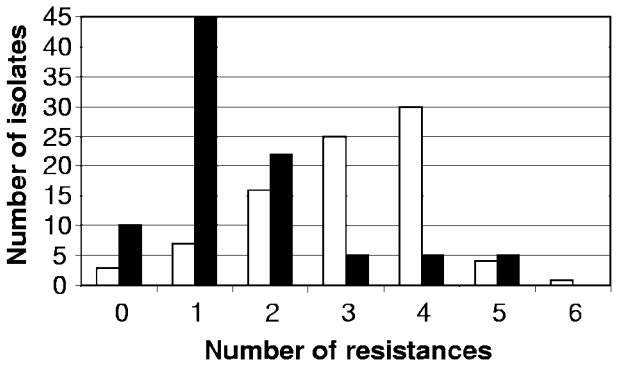

FIG. 4. Number of additional resistances besides *mecA* and *blaZ*. Additional resistances counted were trimethoprim-sulfamethoxazole, tetracycline, chloramphenicol, gentamicin, ciprofloxacin, erythromycin, and rifampin. White bars, MRCNS; black bars, MRSA.

type N1 for the drug clone. The marked difference in the distribution of SCC*mec* profiles in MRSAs and MRCNS suggests that there has not been extensive interspecies SCC*mec* transfer. However, it would be interesting to search for and analyze commensal MRCNS in isolates from drug addicts who are carriers of the drug clone to see if there is SCC*mec* type N1 transfer in that collective of patients.

The degree of multiresistance was clearly higher in MRCNS than in MRSAs (Fig. 4), when resistance to trimethoprim-sulfamethoxazole (SXT), tetracycline, chloramphenicol, gentamicin, ciprofloxacin, erythromycin, and rifampin was determined. The high number of MRSA isolates with no or only one additional resistance determinant was mainly due to the high proportion of SCC*mec* type IV and drug clone MRSA isolates in our strain collection.

Antibiotic resistance in MRSA. (i) SXT. The SXT combination blocks the synthesis of folate derivatives (33). Resistance to trimethoprim in *S. aureus* is formed by mutations in the chromosomal gene for dihydrofolate reductase or by acquisition of the transposon Tn*4003*-borne *dfrA* gene (8, 34). Sulfonamides, competitive inhibitors of dihydropteroate synthase, block folate biosynthesis. Resistance to sulfonamides in staphylococci is due to mutations in the chromosomal dihydropteroate synthase gene (38). The use of the inexpensive SXT combination in the treatment of infections in intravenous drug users may have been one of the driving forces for the association of *dfrA* with the SCC*mec* type N1 element. Although all drug clones amplified the *dfrA* gene, only approximately half of them were resistant to the SXT combination (Table 1). Interestingly, the SXT-susceptible drug clones were generally ciprofloxacin resistant instead, with two exceptions: one isolate was susceptible to both drugs, and one isolate was resistant to both drugs.

The integration of the *dfrA* gene was unique for type N1 SCC*mec* and has not been found in any of the other SCC*mec* types of MRSA and MRSCN analyzed so far. Two STX-resistant isolates with SCC*mec* type IV carried a *dfrA* gene unlinked to SCC*mec*, and 10 SXT-resistant MRSAs did not amplify any *dfrA* gene, suggesting that their SXT resistance was due to chromosomal mutations.

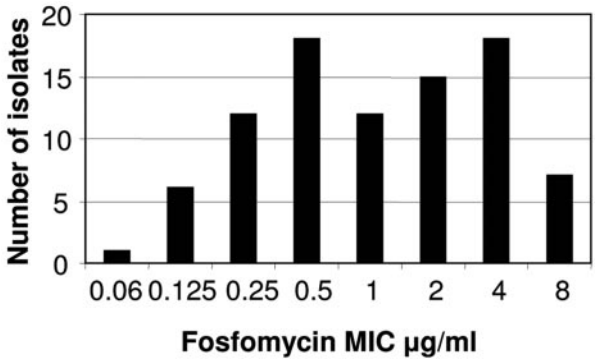

FIG. 5. Distribution of fosfomycin resistance in MRSA.

(ii) Glycopeptides. Glycopeptides sterically inhibit cross-linking and polymerization of the cell wall peptidoglycan by binding to the D-Ala-D-Ala of the nascent peptidoglycan precursor at the cell membrane. VISAs have been found to be the cause for glycopeptide therapy failure in several instances, especially in infections with high bacterial load, but their frequency and relevance have been questioned (35, 41). All MRSAs were screened for glycopeptide resistance upon isolation from patients and prior to storage at −80°C, by using the macro-Etest method that is indicative for potential hVISA (40). A few strains showed elevated teicoplanin and/or vancomycin MICs of >4 mg/ml. However, upon retesting after some months' storage at −80°C, resistance values had dropped, suggesting that glycopeptide resistance was unstable or that lower-resistance variants survived storage at low temperatures better. Only three strains out of all potential hVISAs could be confirmed by population analysis with vancomycin to be similar to that of strain Mu3 (data not shown). One of them belonged to the epidemic ST217 clone, one belonged to a multiresistant type IV isolate, and one belonged to the multiresistant SCC*mec* type N4 isolate.

(iii) Fosfomycin. Fosfomycin inhibits the MurA enzyme, preventing the formation of *N*-acetylmuramic acid, a precursor of the cell wall peptidoglycan. All MRSAs were susceptible to fosfomycin with MICs below the lower fosfomycin breakpoint of 16 mg/ml (24). However, the distribution of fosfomycin MICs suggested the presence of two populations of fosfomycin-susceptible strains in the MRSA collection, namely, isolates with a fosfomycin MIC of around 0.5 and a slightly more resistant population of strains with an MIC of 4 (Fig. 5). The increased fosfomycin MIC correlated with elevated initial teicoplanin MICs (Kendall's rank correlation coefficients: k-tau-a = 0.3346 and k-tau-b of 0.4083; $P < 0.001$). This may be due to upregulation of the *murA* gene, which upon overexpression can confer some fosfomycin resistance. Whether the fosfomycin MIC can be used as indication for upregulated cell wall synthesis in hVISA has to be analyzed further.

(iv) MLS. The structurally different, but functionally similar MLS_B class of drugs binds to the 50S ribosomal subunit, blocking protein synthesis (37). The *erm*-encoded methylases are the most frequent resistance mechanisms against macrolides in staphylococci (3). The inability of lincosamides to induce MLS_B resistance results in clindamycin susceptibility, while constitutive expression of *erm* genes confers resistance to all MLS_B antibiotics. Over 30% (34/90) of the MRSAs had an intermediate level of erythromycin resistance. Inducible MLS_B resistance was found in 17/91 isolates, and constitutive expression was found in 20/91 MRSA isolates.

(v) Linezolid. Linezolid is the first representative of oxazolidinones, a new class of antibiotics which inhibits the assembly of a functional initiation complex for bacterial protein synthesis. It shows no cross-resistance with existing antibiotic agents with the same target (6); in our collection, all MRSAs (MIC at which 50% of the isolates tested are inhibited [MIC_{50}] = 0.5; MIC at which 90% of the isolates tested are inhibited [MIC_{90}] = 1) and MRCNS (MIC_{50} = 0.75; MIC_{90} = 1) were susceptible to linezolid.

CONCLUSIONS

The rather high prevalence of the drug clone, a phenotypically oxacillin-susceptible MRSA, in the Zürich area is challenging, especially since it belongs to the same genetic background as the successfully spreading Berlin clone (42). Because of its low MIC, the drug clone is difficult to detect. The evolution and spread of this clone have to be monitored closely to prevent the clone's escape into other patient populations, where it may pick up other resistance determinants and increase its resistance spectrum. ST45 can harbor different SCC*mec* types. Here, it has acquired a new SCC*mec* type N1, which differs from those previously reported (type II and type IV) to be associated with ST45. The core genetic background of the drug clone is related to the epidemic Berlin clone. Two of its interesting characteristics are its rapid growth rate and the extremely low level of oxacillin resistance, the cause of which is under investigation. Interestingly, we found in this small survey no MRCNS with an SCC*mec* type N1 like that of the drug clone, suggesting that there may be not such a high rate of SCC*mec* exchange between MRCNS and *S. aureus*. Unexpectedly, the MRCNS seemed to harbor multiple new types of SCC*mec* and were more likely to amplify more than one representative of different *ccr* complexes, suggesting that the MRCNS may nevertheless be the breeding ground for new SCC*mec* elements.

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Fitness Cost of Staphylococcal Cassette Chromosome *mec* in Methicillin-Resistant *Staphylococcus aureus* by Way of Continuous Culture[▽]

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We examined the effect of introducing type I or IV staphylococcal cassette chromosome *mec* (SCC*mec*) elements on the growth yield of *Staphylococcus aureus* in glucose-limited continuous culture. Type I showed increased glucose consumption and ATP demand per gram of cells synthesized and decreased cell yield compared to those of the parent strain. In contrast, type IV SCC*mec* elements had no adverse energetic effect.

New strains of methicillin-resistant *Staphylococcus aureus* (MRSA), have emerged in the community, causing infections in young, otherwise healthy people, such as children and military personnel (15, 16), and these strains can achieve a higher infection burden than nosocomial strains (10). In New Zealand, a community-acquired MRSA (CA-MRSA) strain designated Western Samoan phage pattern led to a 15-fold increase in MRSA cases between 1993 and 2000 (14).

The origins of CA-MRSA strains remain elusive. Compared to hospital-acquired MRSA, CA-MRSA strains appear to have higher growth rates, but the basis for this is unknown (11). These strains compete in an environment that is largely devoid of antibiotic selective pressure, and therefore, the selection for factors that contribute to ecological fitness may outweigh the need for multiple resistance determinants. Moreover, genetic factors seem to determine the permissiveness of *S. aureus* strain lineages to accommodate *mecA* and maintain methicillin resistance (8).

In this study, we sought to determine the fitness cost of the staphylococcal cassette chromosome *mec* (SCC*mec*) elements by measuring various physiological parameters between three isogenic *S. aureus* strains carrying either SCC*mec* type I or SCC*mec* type IV elements or no SCC*mec* element, by way of glucose-limited continuous culture.

The susceptible parent strain BB255 is of NCTC8325 background. Strain RA120 is BB255 transformed with an SCC*mec* type I element (4). Strain RA2 was constructed in this study by transformation of strain BB255 with chromosomal DNA obtained from ST92/398, a CA-MRSA isolate harboring a SCC*mec* element type IV (1), as described earlier (4). In vitro transformation frequencies were extremely low ($\leq 4 \times 10^{-1}$/μg of chromosomal DNA). One representative transformant from eight analyzed, designated RA2, was chosen for further study. Figure 1 illustrates that the SmaI-G fragment of strain BB255 harboring *orfX*, the integration site for SCC*mec*, shifted by about 25 kb in the transformant RA2 due to the integration of the SCC*mec* type IV element, which was confirmed by Southern blotting with a *mecA* probe and by SCC*mec* typing (5, 12, 13).

Growth experiments were performed in a medium containing the following: $Na_2HPO_4 \cdot 2H_2O$, 6.0 g/liter; KH_2PO_4, 3.0 g/liter; NaCl, 0.5 g/liter; NH_4Cl, 5.0 g/liter; $CaCl_2$, 15 mg/liter; $MgSO_4$, 247 mg/liter; $MnSO_4$, 1.0 mg/liter; citric acid, 0.06 mg/liter; tryptone, 1.0 g/liter; and glucose, 0.9 g/liter. The cell yield for all strains was proportional to the glucose concentration, indicating that this medium was glucose limited. The doubling times for each strain at 37°C were as follows: 39 ± 3 min for BB255, 41 ± 3 min for RA2, and 54 ± 5 min for RA120. On the basis of these results, the SCC*mec* type I element appeared to have a negative effect on the growth rate of BB255 compared to SCC*mec* type IV in batch culture.

To study the roles of the different SCC*mec* elements on bacterial fitness at precisely controlled growth rates, all strains were grown in glucose-limited continuous culture, with a working volume of approximately 350 ml at a dilution rate of 0.1 h^{-1} (doubling time of 6.9 h). The growth medium was inoculated with exponential-phase cells and permitted to undergo batch growth to densities of approximately 0.3 to 0.8 (optical density at 600 nm) with constant agitation at 200 rpm. Concomitant with the initiation of continuous growth, agitation was then increased to 400 rpm to provide adequate aeration during steady-state conditions. The desired dilution rate was maintained for at least four residence times to allow the culture to reach steady state prior to sampling. At each steady state, 90-ml samples were removed and analyzed for residual glucose (UV method; R-Biopharm), end products (3), total viable CFU on LB agar plates, and cell weight (dry weight). After each sampling, the culture was again maintained for at least four residence times to reach steady state prior to replicate sampling. The values reported are the means of two to four independent experiments with triplicate determinations performed per sampling for each parameter measured. The standard experimental error of the mean associated with these determinations is shown.

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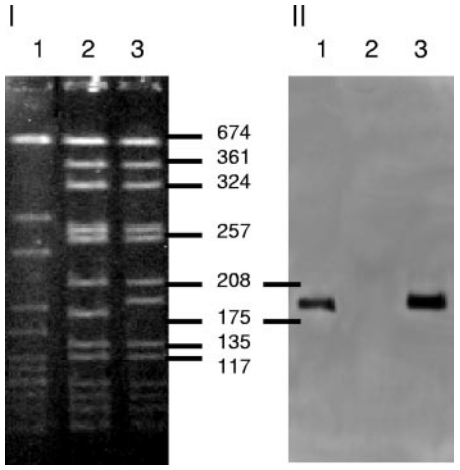

FIG. 1. (I) Pulsed-field gel electrophoresis patterns of Western Samoan phage pattern strain ST92/398 (lane 1), recipient strain BB255 (lane 2), and transformant RA2 (lane 3) after SmaI digestion. (II) Southern hybridization of the pulsed-field gel in panel I with a *mecA* probe. The sizes of the fragments (in kilobases) are shown.

At a dilution rate of 0.1 h^{-1}, cells reached a steady-state optical density at 600 nm of 0.39 to 0.56 (data not shown) and the CFU ranged from 5.75×10^7 to 5.86×10^8 (Fig. 2A). To determine the cell yield on glucose ($Y_{glucose}$), the dry weight and glucose consumption rate of all strains were measured (Fig. 2B and C). Strain RA120 had the lowest dry weight but exhibited the greatest rate of glucose consumption, resulting in the lowest $Y_{glucose}$ for the three strains studied (Fig. 2D). In contrast, strain RA2 had the highest dry weight and the lowest rate of glucose consumption, resulting in a relatively high $Y_{glucose}$. Strain BB255 was intermediate for both parameters. End product analysis from glucose consumption for each strain indicated that glucose was oxidized to CO_2 at each dilution rate, i.e., no incomplete oxidation to acetate or lactate was detected.

When *S. aureus* is grown on glucose as the sole carbon and energy source under aerobic growth conditions, the theoretical ATP yield is 9.4 mol of ATP produced per mol of glucose consumed (6). On the basis of this value, we calculated the weight in grams (dry weight) of cells produced per mole of ATP utilized (Y_{ATP}) for each strain (Fig. 3A). For example, at a dilution rate of 0.1 h^{-1}, the average $Y_{glucose}$ value for strain BB255 was 38.4 g (dry weight) of cells/mol glucose utilized (Fig. 2D) or 38.4 g (dry weight) of cells/9.4 mol of ATP utilized, which is equivalent to a Y_{ATP} value of 4.10 ± 0.3 g (dry weight) cells/mol ATP utilized. The calculated Y_{ATP} value for strain RA2 was 5.64 ± 0.45 g (dry weight) cells/mol ATP utilized, and for RA120, it was 3.24 ± 0.2 g (dry weight) cells/mol ATP utilized (Fig. 3A). These values are equivalent to an ATP demand per gram of newly synthesized biomass for each strain as follows: BB255, 244 ± 18 mmol ATP/g cells synthesized; RA2, 177 ± 22 mmol ATP/g cells synthesized; and for RA120, 308 ± 25 mmol ATP/g cells synthesized (Fig. 3B).

A number of studies have suggested that the size of the SCC*mec* element plays a major role in the fitness of *S. aureus* strains (2, 7, 16). Our data suggest that the SCC*mec* type IV

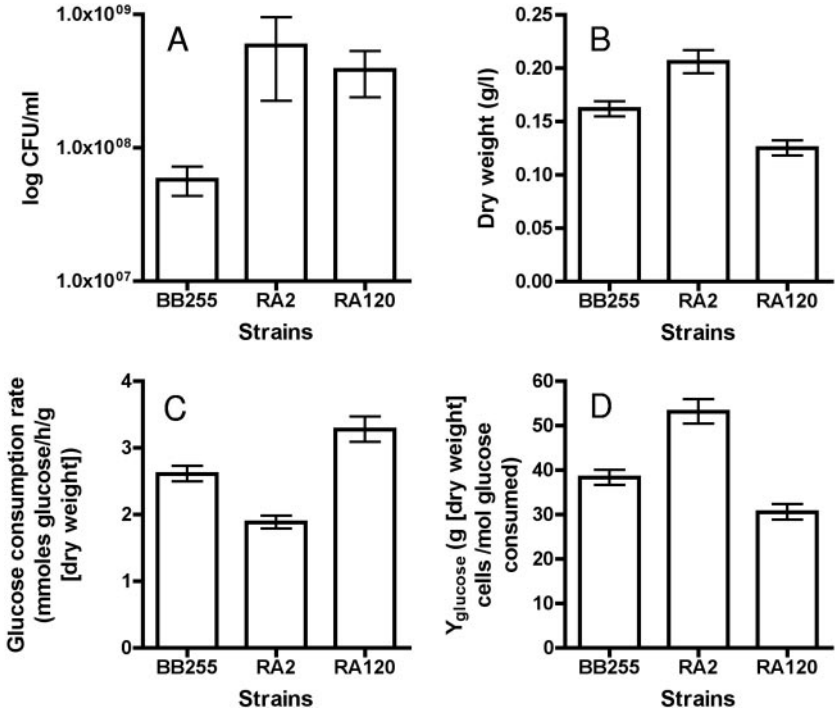

FIG. 2. Glucose-limited continuous culture of *S. aureus* strains at a dilution rate of 0.1 h^{-1}. Strain BB255 is the susceptible parent, RA2 is the BB255 strain transformed with SCC*mec* type IV element, and RA120 is the BB255 strain transformed with SCC*mec* type I element. (A) Log CFU/ml, (B) cell weight (dry weight), (C) glucose consumption rate, and (D) $Y_{glucose}$. The values reported are the means of two to four independent experiments with triplicate determinations performed per sampling for each parameter measured. The standard experimental error of the mean (error bars) associated with these determinations is shown.

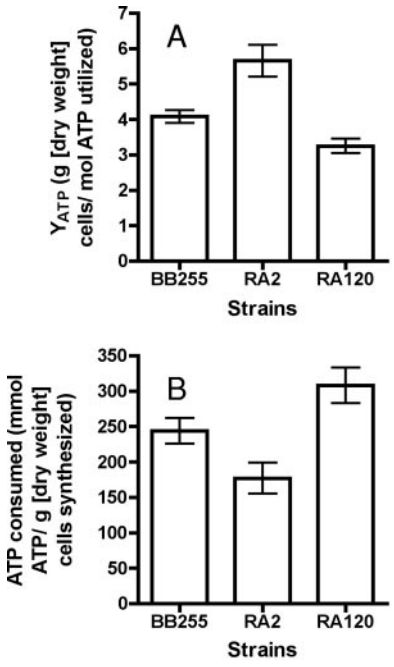

FIG. 3. (A) Y_{ATP} and (B) ATP consumed per gram of newly synthesized biomass for *S. aureus* strains BB255, RA2, and RA120 grown in glucose-limited continuous culture at a dilution rate of 0.1 h^{-1}. Calculations are based on data presented in Fig. 2D and a theoretical ATP yield of 9.4 mol of ATP produced per mol of glucose consumed (6).

element from CA-MRSA did not impose an energetic cost to its naïve host in terms of the maximum growth rate, cell yield, and the amount of cells that can be produced per mole of ATP consumed. However, the SCC*mec* type I element reduced the fitness of its host in terms of growth rate and cell yield. It is unlikely that the difference in size of the SCC*mec* elements is responsible for decreased fitness. Genes expressed from this DNA, such as *pls* coding for the plasmin-sensitive cell wall protein Pls, located on type I but not on type IV SCC*mec* elements, may affect the performance of MRSA. Notwithstanding this, the most likely factor may be the ease at which PBP2a was integrated into the existing cell wall synthesis complex, and the possible secondary compensatory events accompanying this event. Naïve cells, such as strain BB255 used here as a recipient for SCC*mec*, represent a host barrier for PBP2a, and were postulated to have to adapt in order to accept PBP2a (9). The altered ribosome binding site of *mecA* in the type IV SCC*mec* element (unpublished results) predicts lower PBP2a production compared to type I *mecA*, the latter of which is known to produce large and constitutive amounts of PBP2. It is possible that differences in the levels of PBP2a may have triggered different compensatory events and that these have caused the negative effect of SCC*mec* type I elements on growth yield.

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Mosaic Staphylococcal Cassette Chromosome *mec* Containing Two Recombinase Loci and a New *mec* Complex, B2▽

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A novel staphylococcal cassette chromosome (SCC) *mec* from a clinical methicillin-resistant *Staphylococcus aureus* isolate (ST100/CC5) had a mosaic structure, composed of SCC DNA from several different backgrounds. It harbored two complete *ccr* loci and a new variant of *mec* complex B, with Δ*mecR1* interrupted by the aminoglycoside resistance transposon Tn*4001*.

Methicillin resistance in *Staphylococcus aureus* (MRSA) is facilitated by the acquisition of the staphylococcal cassette chromosome *mec* (SCC*mec*), which integrates site specifically into the staphylococcal genome and carries *mecA*, encoding the alternative penicillin-binding protein PBP2a, a β-lactam-insensitive transpeptidase (6, 11, 13, 22, 26). The precise excision and site- as well as orientation-specific integration of this element depend on the action of cassette chromosome recombinase genes (*ccr*'s) located within the element (13).

Five main types of SCC*mec* have been described so far, each differing in size and composition and characterized according to its type of *ccr* locus and *mec* complex (3, 7, 25). *mec* complexes differ in the extents of insertion sequence (IS)-mediated deletions in the *mecA* regulatory genes *mecR1* and *mecI* and the presence and location of insertion sequence IS*431*, IS*1182*, or IS*1272* (23). Apart from the *ccr* and *mec* complexes, and some common mobile resistance elements, SCC*mec* subtypes harbor variable J (junkyard) regions containing truncated and nonessential genes and genes of unknown functions (8). In addition to the major types, a number of new SCC elements, including non-*mecA*-carrying cassettes, have recently been discovered (4, 5, 9, 14, 15, 17, 19).

An epidemiological study of methicillin-resistant staphylococci from Zurich in 2003 identified several strains which contained multiple *ccr* loci (21). Here, we describe the SCC*mec* of one of these isolates, $MRSA_{ZH47}$.

$MRSA_{ZH47}$ is of multilocus sequence type 100 and belongs to clonal complex 5, a genotype previously identified in Argentina (1, 24). In addition to its β-lactam resistance, it was resistant to aminoglycosides and carried a *blaZ*-encoded penicillinase. Its SCC*mec* type could not be determined by standard multiplex PCR (20), and additional *ccr* typing indicated that it contained both *ccr2* and *ccrC* loci (21).

Southern hybridization of SmaI-digested chromosomal DNA, separated by pulsed-field gel electrophoresis, showed that *mecA*- and *ccr2*-hybridizing sequences were colocated on a separate SmaI fragment from the *ccrC*-hybridizing sequence (data not shown).

Transient overexpression of *ccrAB2*, facilitating the precise excision of all major SCC*mec* types (10, 12, 13, 18), was used to cure $MRSA_{ZH47}$. Southern hybridization showed that the resulting oxacillin-susceptible clone $MRSA_{ZH47}$c had lost *mecA* and both *ccr* loci, indicating that all three were present on a single excisable SCC element containing an internal SmaI restriction site (data not shown). The susceptibility profile of $MRSA_{ZH47}$c showed that aminoglycoside resistance had also been lost.

A cosmid library of $MRSA_{ZH47}$ DNA, consisting of over 600 clones with estimated inserts of about 45 kb, was constructed using a SuperCos1 cosmid vector kit (Stratagene, La Jolla, CA). Screening of the library by colony blot analysis using *ccr2*- or *ccrC*-specific probes identified 11 clones that hybridized to *ccr2*, 7 that hybridized to *ccrC*, and 2 that hybridized to both probes. Cosmids were end sequenced and the sequences compared to the genome sequence of *S. aureus* Mu50, revealing that the two cosmids hybridizing with both probes each contained one end of the SCC*mec* element and together completely covered it (data not shown).

Primers specific for known *orfX*, *ccrC2*, IS*431*, and *mecA* nucleotide sequences were used to synthesize long-range PCR products that were subcloned into either pUC19 or pBluescript SK(+). Inserts were end sequenced and the obtained sequences assembled. The double-stranded nucleotide sequence of the 33.7-kb element was completed by primer walking.

This SCC*mec* proved to be unique, containing elements and properties not previously described. GeneMark.hmm (16) and BLASTX (2) identified 33 open reading frames (ORFs), all of which were identical or highly similar in sequence to previously annotated staphylococcal genes (Table 1 and Fig. 1).

The *orfX* insertion site and the characteristic terminal inverted and direct repeats, generated upon insertion, were almost identical to those of other, previously described SCC*mec* elements (12). However, the left-end (proximal) direct repeat sequence contained a nucleotide transition of an adenine to guanine (Table 1), which has not been found elsewhere and increased the identity between the junctional direct repeats. This mutation did not impede the excision of the element, as demonstrated by the precise curing of $SCCmec_{ZH47}$ from the chromosome of $MRSA_{ZH47}$. It could, however, possibly influence the stability or transfer frequency of the element.

$SCCmec_{ZH47}$ contained a new *mec* complex that we have

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TABLE 1. ORFs encoded on SCC*mec*$_{ZH47}$

Coding sequence no.	Name[a]	Position (bp)	Identity (%)[b]	Homolog(s)[c]	Information[d]
	DR-L	754–771			Integration site sequence of SCC*mec*, A→G substitution at position 16 of left direct repeat
	IR-L	757–764			Integration site sequence of SCC*mec*; left inverted repeat
1	ZH02	1017–1322	98	CZ078 (85/2082)	Hypothetical protein, predicted restriction endonuclease domain (COG 3183)
2	ZH03	1887–2384	100	CZ077 (85/2082)	Conserved hypothetical protein (COG 3680)
3	ZH04	2468–3967	100	CZ076 (85/2082)	Hypothetical protein
4	ZH05	4193–5293	100	CZ075 (85/2082)	Hypothetical protein; DNA polymerase A family domain (Pfam 00476.12)
5	ZH06	5286–5657	94	CZ074 (85/2082)	Hypothetical protein (DUF 1092)
6	ZH07	5654–7273	100	3 half: CG008 (85/3907)	Hypothetical protein; POX_D5 domain associated with viral DNA replication (Pfam 03288.11)
			100	5 half: unnamed ORF (TSGH17)	
7	*ccrC*	7498–9174	94	*ccrC2* (TSGH17)	Cassette chromosome recombinase C
			89	*ccrC3* (85/2082)	
8	ZH09	9280–9618	98	SSP0034 (ATCC 15305)	Hypothetical protein
9	ZH10	9714–10025	90	SSP0032 (ATCC 15305)	Hypothetical protein, contains SmaI restriction site
10	ZH11	10041–10547	90	CZ068 (85/2082)	Conserved in gram-positive and -negative bacteria but of unknown function (COG 4333)
11	IS431	10696–11370	99	IS*431* (SCC*mec* types I–IV)	Insertion sequence IS*431*
12	ZH13	11628–11795	100	ORF CN041 (N315) and in SCC*mec* types I–V	Putative HMG-coenzyme A-synthase (cholesterol biosynthesis)
13	*ugpQ*	12712–13455	100	*ugpQ* (all SCC*mec* types)	Glycerophosphoryl diester phosphodiesterase
14	ZH15	13552–13980	100	SA0037 (N315) and in SCC*mec* types I–V	Hypothetical protein, MaoC-like domain (Pfam 01575.11)
15	*mecA*	14026–16032	100	WIS (SCC*mec* type V) and TSGH17 (SCC*mec* type V_T)	Penicillin-binding protein PBP2a
16	Δ*mecR1′*	16132–16958	100	Δ*mecR1* from *mec* complex B	First 826 bp of Δ*mecR1*, truncated signal transducer MecR1 from *mec* complex B
	DR_{Tn4001}	16951–16958			Repeated region generated by transposon integration
	IR_{Tn4001}	16959–17059			Repeated region generated by transposon integration
17	IS256L	17060–18232	100	Tn*4001*	IS of Tn*4001*
18	*aac*	18235–18681	100	Tn*4001*	Putative *N*-acyltransferase, GNAT family
19	*aac*(2′)–*aph*(6″)	18682–20121	100	Tn*4001*	Aminoglycoside-(2′)-acetyltransferase-aminoglycoside-(6″)-phosphotransferase
20	IS256R	20251–21423	100	Tn*4001*	IS of Tn*4001*
	IR_{Tn4001}	21424–21524			Repeated region generated by transposon integration
	DR_{Tn4001}	21525–21532			Repeated region generated by transposon integration
21	Δ*mecR1″*	21533–21692	100	Δ*mecR1* from *mec* complex B	Remainder of Δ*mecR1*, interrupted by Tn*4001* insertion
22	ZH22	21595–21924	100	Δ*hsdR* (MW2)	Truncated hypothetical protein, similar to type I restriction endonuclease
23	ZH23	21915–23438	100	IS*1272* transposase from *mec* complex B	aa 1–102: transposase and inactivated derivatives (COG 3666)
					aa 185–459: transposase DDE domain
24	ZH24	23574–24083	100	Various type IV SCC*mec*s	Hypothetical protein
25	ZH25	24095–24406	100	Various type IV SCC*mec*s	Hypothetical protein
26	ZH26	24493–24843	100	Various type IV SCC*mec*s	Hypothetical protein
27	*ccr2B*	25365–26993	100	*ccrB* SCC*mec* type IVd (JCSC4469)	Cassette chromosome recombinase B
28	*ccr2A*	27015–28364	100	*ccrA* SCC*mec* type IVe (AR43)	Cassette chromosome recombinase A
29	ZH29	28598–30391	100	R004 (MR108)	aa 1–382: superfamily II helicase and inactivated derivatives (COG 5519)
30	ZH30	30391–30687	100	Different type IV SCC*mec*s	Hypothetical protein
31	ZH31	30880–31926	100	Different type IV SCC*mec*s (ATCC 12228)	Hypothetical protein
32	ZH32	32381–33535	100	CR008 (MR108)	aa 1–122: *abi* alpha protein, predicted transcriptional regulator (COG 2865)
33	ZH33	33565–34365	100	Different type IV SCC*mec*s and SE0042 (ATCC 12228)	Hypothetical protein, abortive phage resistance protein
	IR-R	34423–34430			Integration site sequence of SCC*mec*; right inverted repeat
	DR-R	34432–34449			Integration site sequence of SCC*mec*; right direct repeat

[a] ORFs of known functions are named accordingly; putative and hypothetical ORFs have been assigned ZH numbers.
[b] Amino acid sequence identity.
[c] The name of the homologous gene is indicated, and the strain in which it was found is indicated in parentheses. If homologous ORFs were unnamed, the SCC*mec* type in which they were found is indicated.
[d] Information about the element, e.g., about the encoded protein. Positions and reference numbers of known protein domains are indicated. aa, amino acids; HMG, 3-hydroxy-3-methylglutaryl; COG, cluster of orthologous groups of proteins.

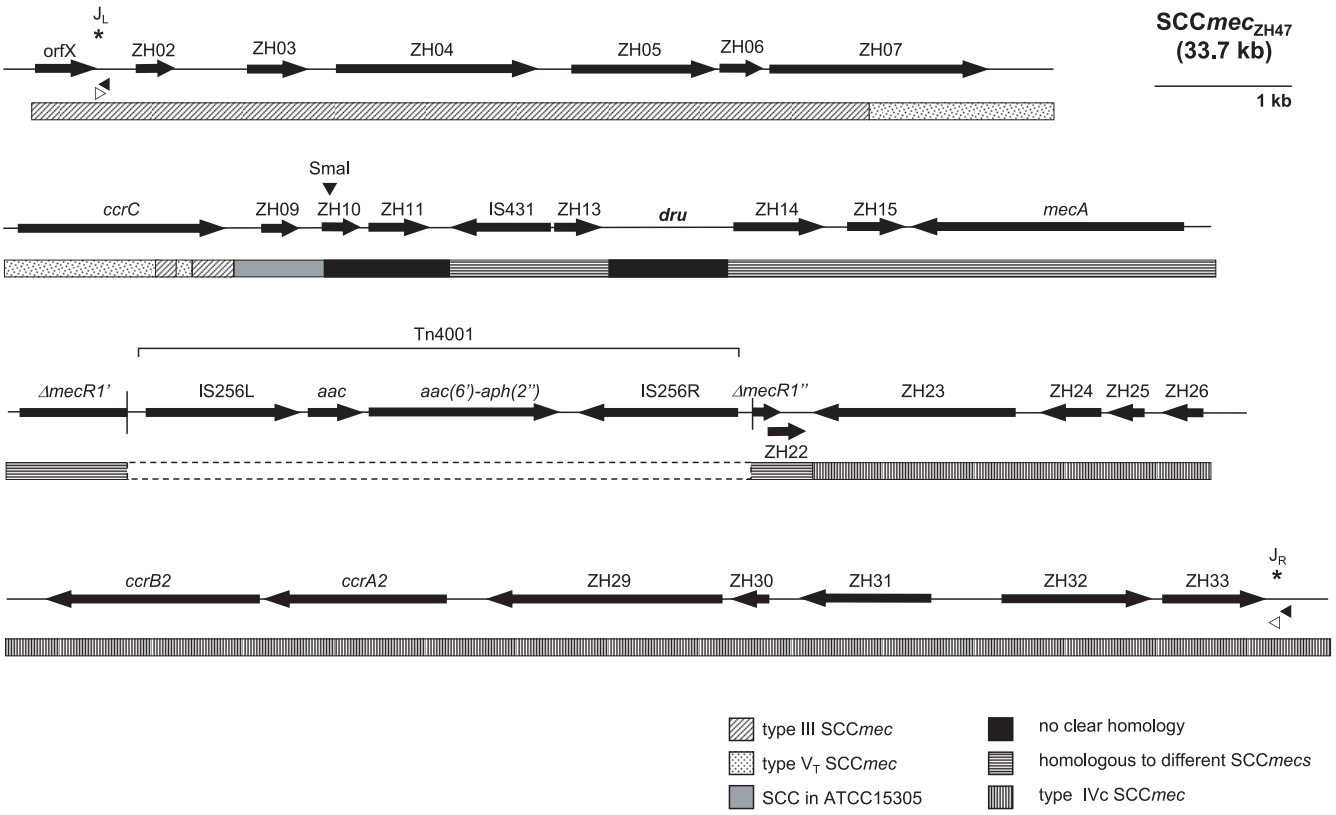

FIG. 1. Genetic map of the mosaic SCC*mec* element from MRSA$_{ZH47}$. Open reading frames are symbolized by arrows, and the bars below them indicate homologies to previously described SCC elements. Integration site sequences for SCC*mec* (*), direct repeats of chromosomal junctions (◀), and inverted repeats (▷) are indicated. J_L denotes the junction proximal and J_R the junction distal to the origin of replication.

named B2 because of its similarity to *mec* complex B. The new B2 complex differed in that the 987-bp Δ*mecR1* fragment was interrupted by insertion of the aminoglycoside resistance transposon Tn*4001* at base pair position 820 (Fig. 1), and while the *mecA* promoter region was identical to that of *mec* complex B, the *mecA* gene sequence was identical to that of SCC*mec* types V and V_T.

In addition to a *ccrAB2* locus at the usual position downstream of *mecA*, the element possessed a *ccrC* locus between *orfX* and the *dru* element (Fig. 1). The *ccrA2* sequence was identical to that of SCC*mec* type IVe, while the *ccrB2* sequence was identical to that of SCC*mec* type IVd. Comparison of the *ccrC* sequence to published variants revealed high levels of similarity to the *ccrC2* and *ccrC3* sequences of SCC*mec* types V_T and III, respectively (Table 1).

The element as a whole appeared mosaic in structure. The presence of both a *ccrAB2* locus and a variant *ccrC* locus and of regions with strong similarity to several different SCC elements, including the typical hospital-acquired MRSA type III SCC*mec*, the community-associated MRSA SCC*mec* types IV and V_T, and SCC*mec* from the non-*S. aureus* species *Staphylococcus saprophyticus*, suggests that SCC*mec*$_{ZH47}$ had been assembled via several recombination events (Fig. 1).

Most of the new SCC and SCC*mec* elements recently discovered, including the SCC*mec*$_{ZH47}$ described here, appear to have acquired regions from other SCC elements, suggesting that significant intra- and interspecies exchange and recombination of SCC DNA occurs.

Nucleotide sequence accession number. The nucleotide sequence newly determined in this study was deposited in the EMBL database under accession number AM292304.

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